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14. ABSTRACT

Metastases are responsible for >90% of human cancer deaths; yet, our knowledge regarding the molecular regulation of metastasis remains fragmentary. MicroRNAs are short RNAs that suppress their targets post-transcriptionally, leading to modulation of diverse biological processes. MicroRNAs are well-suited to regulate metastasis due to their capacity to concomitantly inhibit numerous target genes. We have identified a microRNA, miR-31, whose levels correlated inversely with metastatic recurrence in human breast cancer patients. Expression of miR-31 in otherwise-aggressive breast tumor cells impeded metastasis. We deployed a novel microRNA sponge strategy to stably inhibit miR-31; this allowed otherwise-non-aggressive breast cancer cells to metastasize. These phenotypes were achieved via pleiotropic modulation of a cohort of clinically relevant metastasis-promoting genes, which were overrepresented among the >200 mRNAs predicted to be direct targets of miR-31. In fact, we discovered that miR-31-evoked concurrent regulation of three such effectors – integrin α5, radixin, and RhoA – was sufficient to explain this microRNA's impacts on metastasis. Together, these findings provide insights into metastasis that may prove useful in the diagnosis and/or treatment of breast cancer.

15. SUBJECT TERMS

Breast cancer; tumor metastasis; cancer; miR-31; microRNAs and disease

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INTRODUCTION

Metastases, as opposed to primary tumors, account for 90% of human cancer deaths[1,2]. In order to metastasize, cells in a primary tumor must become motile, degrade surrounding extracellular matrix (local invasion), intravasate into the vasculature, retain viability during transit through the circulation, extravasate into the parenchyma of a distant tissue, survive in this foreign microenvironment to form micrometastases, and finally thrive in their new milieu and establish macroscopic secondary tumors (colonization)[1,2]. It has recently been appreciated that, in addition to mutations in protein-encoding genes, deregulation of non-coding RNAs also causatively elicits malignancy[3-5]. MicroRNAs (miRNAs), pleiotropically acting endogenous small RNAs that post-transcriptionally silence gene expression via specific interaction with the 3' UTR of cognate targets[6-8], function as both oncogenes and tumor suppressor genes[3-5]. However, the contributions of specific miRNAs to the core circuitry of breast cancer metastasis remain largely unresolved[3,5]. This training grant endeavors to further our knowledge regarding the role of specific miRNAs in controlling breast cancer metastasis by (1) identifying metastasis-relevant miRNAs and (2) deciphering the molecular means by which such miRNAs regulate metastatic progression. We anticipate that these findings will prove significant for our basic understanding of the mechanistic underpinnings of tumor metastasis, as well as elucidate putative diagnostic and/or therapeutic targets in human breast cancer.

BODY

Task #1: Execute the detailed facets of my training plan (Months 1-36). During the first year of this award, I have succeeded in undertaking all of the sub-tasks detailed previously in this specific aim, including performing laboratory research, completing my teaching responsibilities with the MIT Department of Biology, actively participating in weekly laboratory group meeting (including eight occasions on which I was responsible for presenting my research findings), actively participating in daily laboratory journal clubs (including 12 instances where I was responsible for presenting a primary research article to my colleagues), attending departmental seminars hosted by the MIT Department of Biology, participating in weekly intra-departmental "Cancer Super-Group" seminars (including presenting my research findings on two different occasions), and by attending one scientific conference related to the subject of breast cancer metastasis.

Task #2: Ascertain whether miR-31 suppresses breast cancer metastasis *in vivo* (Months 1-24). The findings of these experiments provided the basis for our recent publication describing the role of miR-31 in breast cancer metastasis[9] (please refer to Figures 2-3 in ref. 9, provided in the appendix, for further details). At present, we are attempting to further extend these observations by assaying the ability of miR-31 to impair the outgrowth of already-disseminated tumor cells. To do so, we are employing doxycycline-inducible miR-31 expression vectors and doxycycline-repressible miR-31 miRNA sponge constructs. We anticipate that the findings of these experiments may provide information that will assist in determining whether miR-31 is likely to represent an attractive therapeutic target for intervention therapies in human breast cancer patients.

Task #3: Elucidate downstream effectors of miR-31 (Months 1-12). The findings of these experiments were a pivotal component of our recent publication describing the role of miR-31 in breast cancer metastasis[9] (please refer to Figure 4 in ref. 9, provided in the appendix, for further information). At present, we are further expanding upon our previous observations by

deploying microarray gene expression profiling technology as an alternative means by which to implicate specific mRNAs as downstream targets of miR-31 in human breast cancer cells.

Task #4: Identify regulatory factors that function directly upstream of miR-31 (Months 13-24). This specific aim represents one of our key goals for the upcoming award period. Given the critical role that we have established for miR-31 function during metastatic progression in breast cancer[9,10], we imagine that comprehension of the means by which the expression levels of this miRNA are regulated will afford significant insight into tumor development. Moreover, our findings may identify potential diagnostic and/or therapeutic targets for metastatic human breast cancer. To this end, we will utilize both computational and experimental approaches to investigate regulatory factors that function directly upstream of miR-31 to control its expression. These analyses will include interrogation of the epigenetic events that contribute to miR-31 repression in metastatic human breast cancer cells.

Task #5: Highlight the niche of miR-31 within metastatic core signalling circuitry (Months 13-36). The findings of these experiments were integral components of two of our recent publications describing the role of miR-31 in breast cancer metastasis[9,10] (please refer to Figures 5-6 in ref. 9 and Figures 1-4 in ref. 10, provided in the appendix, for further information). At present, we are further expanding upon these prior observations by (1) creating otherwise-metastatic human breast cancer cells that concomitantly express various combinations of shRNAs targeting functionally relevant downstream targets of miR-31 and assaying the metastatic capacities of these cells *in vitro* and *in vivo*, as well as (2) completing these *in vitro* and *in vivo* assays using breast cancer cells expressing either shRNAs or cDNA constructs encoding additional (previously functionally unexplored) direct downstream targets of miR-31. We have elected to focus our attention upon genetic means by which to assess the functional relevance of implicated miR-31 target genes – as opposed to pharmacological inhibitors – as we have discovered that pharmacological inhibition of certain miR-31 effectors is either not currently possible and/or confounded by off-target effects of the inhibitory molecules.

Task #6: Assess the relevance of miR-31 for disease progression in human patients (Months 30-36). Our preliminary findings on this subject have been summarized in our recent publication regarding the association of miR-31 expression with disease outcome in human breast cancer patients[9] (please refer to Figure 7 in ref. 9, provided in the appendix, for further information). At present, we are striving to further validate and extend these observations. To do so, we are working to assemble a larger cohort of human breast cancer patient specimens, within which we will then quantify miR-31 levels and assess any correlations between miR-31 status and patient outcome. In these analyses, we will endeavor to decipher the correlation between miR-31 expression levels and propensity for metastatic recurrence, as well as additional disease parameter of clinical significance.

KEY RESEARCH ACCOMPLISHMENTS

-Demonstrated that miR-31 expression is sufficient to impair metastasis in otherwise-aggressive human breast cancer cells

-Provided evidence that miR-31 expression is necessary to prevent the acquisition of a metastatic phenotype by otherwise-benign human breast cancer cells

-Showed that miR-31 intervenes during at least three distinct steps of the invasion-metastasis cascade (local invasion, one or more early post-intravasation events, and metastatic colonization)

-Identified six clinically relevant direct downstream effectors of miR-31 in metastatic human breast cancer cells
-Elucidated that the re-expression of four of these six downstream targets was capable of reversing, at least partially, miR-31's effects on one or more *in vitro* surrogate markers of metastatic capacity
-Demonstrated that individual restored expression of three of these six downstream target genes was sufficient to partially rescue miR-31-imposed metastasis suppression *in vivo*
-Revealed that concomitant re-expression of three of these downstream effectors was able to entirely reverse miR-31-evoked inhibition of metastasis *in vivo*
-Discovered that miR-31 expression was inversely correlated with propensity for metastatic relapse in human breast cancer patients
-Developed a novel means for the stable suppression of microRNA function *in vitro* and *in vivo* – a tool that is likely to prove useful to a number of researchers within the microRNA community
-Established tools and reagents to facilitate comprehension of several major unresolved questions concerning the role of miR-31 in tumor development and progression

REPORTABLE OUTCOMES

-Manuscripts

1) **Valastyan S**, Reinhardt F, Benaich N, Calogrias D, Szász AM, Wang ZC, Brock JE, Richardson AL, and Weinberg RA. 2009. A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell*. 137: 1032-1046.
2) **Valastyan S** and Weinberg RA. 2009. MicroRNAs: crucial multi-tasking components in the complex circuitry of tumor metastasis. *Cell Cycle*. 8: 3506-3512.
3) **Valastyan S** and Weinberg RA. 2009. Assaying microRNA loss-of-function phenotypes in mammalian cells: emerging tools and their potential therapeutic utility. *RNA Biology*. 6.
4) **Valastyan S**, Benaich N, Chang A, Reinhardt F, and Weinberg RA. 2009. Concomitant suppression of three target genes can explain the impact of a microRNA on metastasis. *Genes and Development*. 23.
5) Ma L, Young JJ, Prabhala H, Mestdagh P, Muth D, Teruya-Feldstein J, Reinhardt F, Onder TT, **Valastyan S**, Westermann F, Speleman F, Vandsompele J, and Weinberg RA. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nature Cell Biology*. Manuscript under revision.

-Presentations

-Whitehead Institute Forum, seminar (12/08)
-Harvard Breast Cancer SPORE Project and Program Grant, seminar (4/09)
–Beatson International Cancer Conference, recipient of Trevigen poster prize (7/09)
-Colrain Cancer Symposium, seminar (10/09)

-Developed numerous cell lines, histological specimens, and other technologies that may be of use to other researchers interested in the topic of breast cancer metastasis

CONCLUSION

The experience and knowledge gained as a result of this training grant have both aided in my personal development as an independent research scientist and furthered the research community's general understanding regarding the mechanistic underpinnings of breast cancer metastasis. More specifically, these endeavors have resulted in the identification of miR-31, a miRNA that functions as a potent suppressor of breast cancer metastasis. Moreover, our studies have elucidated a cohort of clinically relevant downstream effectors of miR-31 whose concomitant suppression by this miRNA are likely to underlie miR-31's observed impact on metastatic dissemination. Of importance is our observation that miR-31 levels were inversely associated with propensity for metastatic relapse in human breast cancer patients; this raises the possibility that miR-31 may come to represent a useful prognostic marker for the diagnosis of breast cancer in a clinical setting. Furthermore, our ongoing investigations concerning the potential benefits of temporally controlled re-introduction of miR-31 into already-disseminated metastatic tumor cells using a murine breast cancer model may further bolster the putative clinical utility of miR-31, as the findings of these assays may suggest that miR-31 might additionally represent an efficacious therapeutic target for breast cancer intervention and remediation. Finally, our future interrogations of the upstream regulatory stimuli that control miR-31 expression levels in aggressive human breast cancer cells may highlight other potentially interesting prognostic and/or therapeutic targets in human breast cancer. Taken together, the studies supported by this training grant have apparent relevance to the diagnosis and treatment of metastatic human breast cancer; in light of the fact that metastases are responsible for greater than 90% of human deaths from breast cancer[1,2], these findings may prove significant to the process of combating this disease.

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APPENDIX

Cell

A Pleiotropically Acting MicroRNA, miR-31, Inhibits Breast Cancer Metastasis

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SUMMARY

MicroRNAs are well suited to regulate tumor metastasis because of their capacity to coordinately repress numerous target genes, thereby potentially enabling their intervention at multiple steps of the invasion-metastasis cascade. We identify a microRNA exemplifying these attributes, miR-31, whose expression correlates inversely with metastasis in human breast cancer patients. Overexpression of miR-31 in otherwise-aggressive breast tumor cells suppresses metastasis. We deploy a stable microRNA sponge strategy to inhibit miR-31 in vivo; this allows otherwise-nonaggressive breast cancer cells to metastasize. These phenotypes do not involve confounding influences on primary tumor development and are specifically attributable to miR-31-mediated inhibition of several steps of metastasis, including local invasion, extravasation or initial survival at a distant site, and metastatic colonization. Such pleiotropy is achieved via coordinate repression of a cohort of metastasis-promoting genes, including RhoA. Indeed, RhoA re-expression partially reverses miR-31-imposed metastasis suppression. These findings indicate that miR-31 uses multiple mechanisms to oppose metastasis.

INTRODUCTION

Metastases account for 90% of human cancer deaths (Gupta and Massagué, 2006), yet our understanding of the molecular circuitry that governs metastatic dissemination remains fragmentary. The invasion-metastasis cascade, which leads to these growths, is a complex, multistep process involving the escape of neoplastic cells from a primary tumor (local invasion), intravasation into the systemic circulation, survival during transit through the vasculature, extravasation into the parenchyma of distant tissues, the establishment of micrometastases, and ultimately the outgrowth of macroscopic secondary tumors (colonization) (Fidler, 2003).

MicroRNAs (miRNAs) constitute an evolutionarily conserved class of pleiotropically acting small RNAs that suppress gene expression posttranscriptionally via sequence-specific interactions with the 3′ untranslated regions (UTRs) of cognate mRNA targets (Bartel, 2009). In mammalian cells, miRNAs effect gene silencing via both translational inhibition and mRNA degradation; an individual miRNA is capable of regulating dozens of distinct mRNAs, and together the >650 human miRNAs are believed to modulate more than one-third of the mRNA species encoded in the genome (Bartel, 2009).

A central role for miRNAs in the establishment and progression of human tumors has begun to emerge. More than 50% of miRNA-encoding loci reside in chromosomal regions altered during tumorigenesis (Calin et al., 2004), and expression profiling reveals characteristic miRNA signatures for many tumor types—including breast neoplasias—that predict disease status and clinical outcome (Calin and Croce, 2006). In addition, miRNAs have been identified that function as classical oncogenes or tumor suppressor genes (Ventura and Jacks, 2009), as well as a limited number that act at late stages of tumor progression (Ma et al., 2007; Tavazoie et al., 2008; Huang et al., 2008; Asangani et al., 2008; Zhu et al., 2008; Lujambio et al., 2008).

The extent to which miRNAs specifically affect metastasis remains unclear, because all the miRNAs reported to affect metastasis also exert potentially confounding influences on primary tumor development, apoptosis, and/or cell proliferation (Voorhoeve et al., 2006; Sathyan et al., 2007; Ma et al., 2007; Si et al., 2007; Tavazoie et al., 2008; Kondo et al., 2008; Lujambio et al., 2008). Moreover, a role for miRNAs in steps of the invasion-metastasis cascade subsequent to local invasion has not been described.

The pleiotropic nature of gene regulation exhibited by miRNAs led us to hypothesize that certain miRNAs might be endowed with a capacity to function as crucial modulators of tumor metastasis. Here, we identify an antimetastatic human miRNA, miR-31, that acts at multiple steps of the invasion-metastasis cascade via repression of a cohort of prometastatic targets.

RESULTS

miR-31 Expression Is Specifically Attenuated in Metastatic Breast Cancer Cell Lines

To identify miRNAs that might regulate breast cancer metastasis, we selected 10 cancer-associated miRNAs for further characterization because of their concordant identification among expression profiling studies of clinical breast tumors (Iorio et al., 2005; Volinia et al., 2006), global analysis of miRNA copy-number variation in human breast carcinomas (Zhang et al., 2006), and localization of miRNA loci to cancer-relevant sites of chromosomal aberration (Table S1 available online; Calin et al., 2004). These studies did not stratify patients based on metastasis status.

Expression of the 10 candidate miRNAs was assayed in 15 human and mouse mammary cell lines, which included normal epithelial cells, tumorigenic but nonmetastatic cells, and metastatic tumor cells (Table S2). The levels of a single miRNA, miR-31, were specifically attenuated in aggressive human breast cancer cells when compared to primary normal human mammary epithelial cells (HMECs). Although nonmetastatic tumor cells (HMLER, MCF7-Ras, and SUM-149) exhibited 4-fold reduced miR-31, expression of this miRNA in metastatic SUM-159 and MDA-MB-231 cells was diminished by >100-fold (Figure 1A).

Relative to its expression in normal murine mammary gland (NMuMG) cells, miR-31 levels in sublines derived from a single murine mammary tumor reflected their capacities to metastasize: miR-31 was reduced by 2-fold in metastatic D2.1 and D2A1 cells, but not in nonaggressive D2.OR cells (Figure 1B). miR-31 levels were also inversely proportional to metastatic ability in four mouse mammary carcinoma sublines derived from a single spontaneously arising tumor: although miR-31 levels in nonaggressive 67NR cells were similar to those in NMuMG, miR-31 expression was progressively diminished upon acquisition of the capacity to invade locally (168FARN), to form micrometastases (4TO7), and to yield macroscopic metastases (4T1) (Figure 1B). Thus, miR-31 levels are specifically attenuated in aggressive breast cancer cells.

miR-31 expression was heterogeneous in 4T1 cell primary mammary tumors; of note, the proportion of cells expressing miR-31 was 10-fold reduced in lung metastases relative to the fraction of miR-31-positive cells in the primary tumors from which they were derived (Figure 1C). Also, 5-fold fewer cells located near the invasive front of 4T1 cell mammary tumors expressed miR-31, compared to cells in the interior of these tumors (Figure 1D). These data raise the possibility that selective pressures diminish the prevalence of miR-31-expressing cells within the pool of successfully metastasizing cells during the course of metastatic progression.

miR-31 Expression Suppresses Metastasis-Relevant Traits In Vitro

Given these inverse correlations between miR-31 levels and malignant phenotypes, we assessed the potential for antimetastatic roles for miR-31. Thus, we stably expressed miR-31 in metastatic MDA-MB-231 human breast cancer cells (“231 cells”). This overexpression resulted in miR-31 levels comparable to those in HMECs (Figure S1A).

Ectopic miR-31 did not affect proliferation in vitro, but did reduce invasion by 20-fold and motility by 10-fold (Figure 2A; Figures S1B and S1C). These effects were specifically attributable to the biological activities of miR-31, as indicated by the fact that equivalent overexpression of a control miRNA, miR-145, failed to influence invasion or motility (Figure 2A and data not shown). Also, miR-31-expressing cells exhibited 60% diminished resistance to anoikis-mediated cell death (Figure 2B).

These defects could not be ascribed to toxicity resulting from ectopic miR-31 (Figure S1D). The consequences of miR-31 expression were not unique to 231 cells: miR-31 reduced invasion, motility, and anoikis resistance, yet did not affect proliferation, in aggressive SUM-159 human breast cancer cells (Figure S2). Hence, miR-31 impairs in vitro surrogates of metastatic ability.

miR-31 Expression Suppresses Metastasis In Vivo

Because of its effects on in vitro traits associated with high-grade malignancy, we asked whether ectopic miR-31 could inhibit metastasis in otherwise-aggressive cells. Thus, 231 cells expressing miR-31 were injected into the orthotopic site—the mammary fat pad—of mice. Unexpectedly, miR-31 enhanced primary tumor growth by 1.5-fold and correspondingly increased cell proliferation (Figure 2C; Figure S3A). Control 231 cell primary tumors displayed evidence of local invasion; however, miR-31-expressing tumors were well encapsulated and noninvasive (Figures 2D and 2E). These changes were not accompanied by altered neovascularization (Figure S3B).

Despite their ability to generate larger primary tumors, 231 cells expressing miR-31 were strikingly impaired in their capacity to seed lung metastases. miR-31-expressing cells formed 95% fewer lesions than did controls 62 days after implantation (Figure 2F). Thus, miR-31 suppresses metastasis from an orthotopic site, ostensibly due, at least in part, to its ability to impede local invasion.

We addressed the possibility that miR-31’s impact on these parameters was attributable to clonal variation in our 231 cells by expressing miR-31 in a single-cell-derived population isolated from the parental 231 cells (Figure S4A; Minn et al., 2005). As before, when injected orthotopically, miR-31-expressing cells formed large, well-encapsulated primary tumors and also reduced lung metastasis by 5-fold (Figures S4B–S4D). Orthotopic injection of SUM-159 cells expressing miR-31 further corroborated our earlier findings: miR-31 enhanced primary tumor growth, yet miR-31-expressing tumors were better confined than control tumors (Figure S5). These observations indicated that the ability of miR-31-expressing cells to form larger, less invasive primary tumors, as well as to seed fewer metastases, is a specific consequence of the biological activities of miR-31.

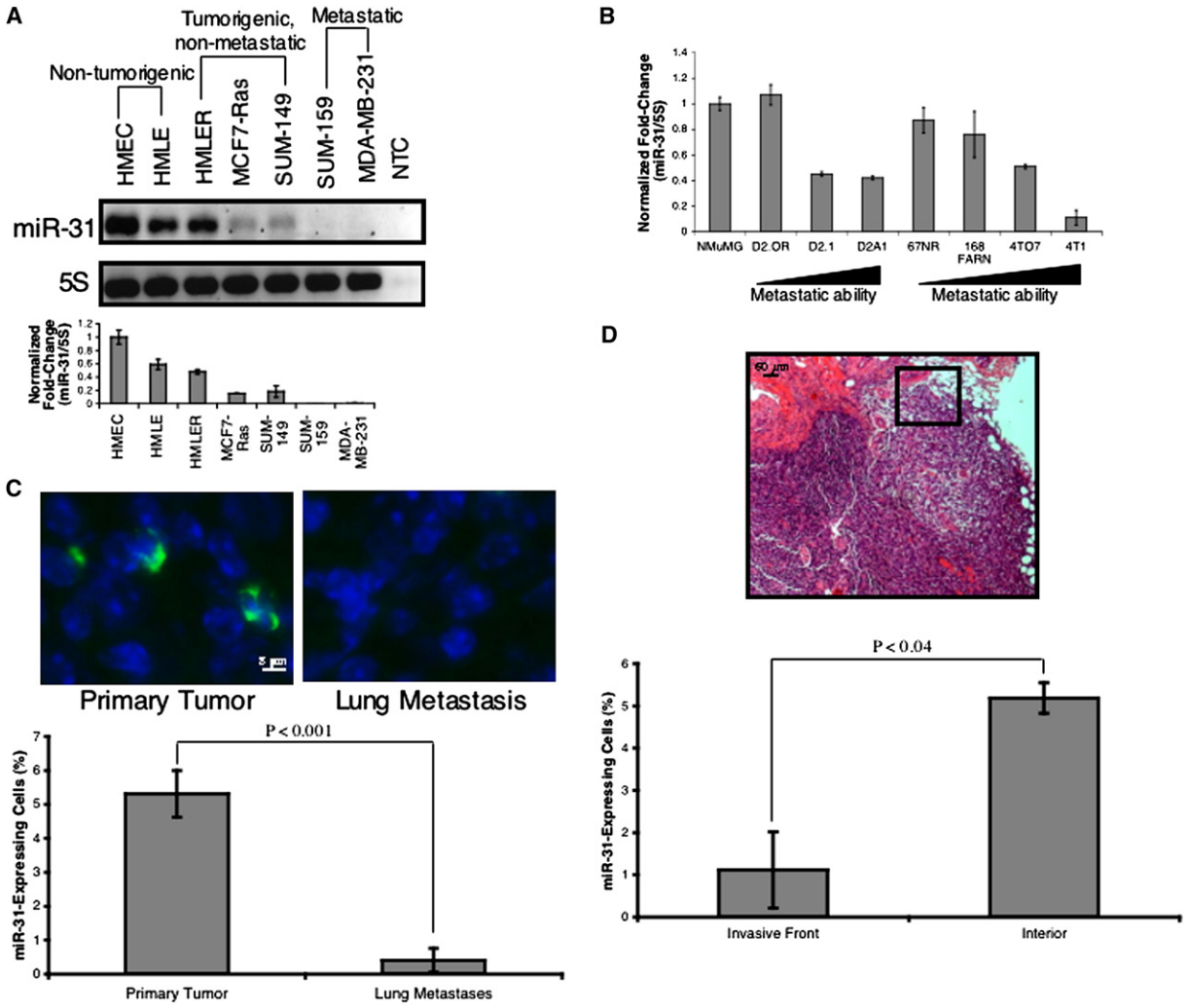

Figure 1. miR-31 Levels Correlate Inversely with Metastatic Ability in Breast Cell Lines

(A) RT-PCR for miR-31 in seven human breast cell lines. 5S rRNA was a loading control. NTC, no template control. n = 3.

(B) miR-31 RT-PCR in eight murine mammary cell lines. 5S rRNA was a loading control. n = 3.

(C) In situ hybridization for miR-31 (green) in animal-matched 4T1 cell primary mammary tumors and lung metastases; DAPI counterstain (blue). n = 4.

(D) Hematoxylin and eosin (H&E) stain of a 4T1 cell primary mammary tumor (top); box: invasive front. miR-31 in situ hybridization in 4T1 cells located near the invasive front or the interior of the primary tumors (bottom). n = 3.

Data are presented as mean ± SEM.

We determined whether miR-31's impact on metastasis was also attributable to effects on later steps of the invasion-metastasis cascade, independent of its influence on local invasion. Thus, we injected miR-31-expressing 231 cells directly into the circulation of mice, thereby circumventing the initial steps of local invasion and intravasation. After 1 day, miR-31-expressing cells were 4-fold impaired in their ability to persist in the lungs (Figure 2G). This difference was not a consequence of an inability of miR-31-expressing cells to become lodged initially in the lung microvasculature, as shown by the fact that equal numbers of miR-31-expressing and control cells were detected in the lungs 10 min and 2 hr after injection (Figure 2G; Figure S6A). These observations suggested that miR-31 regulates early postintravasation events, such as intraluminal viability, extravasation, and/or initial survival in the lung parenchyma.

Three months after tail vein injection, miR-31-expressing 231 cells generated 40-fold fewer lung metastases than did controls (Figure 2G). We also observed a dramatic effect on the size of eventually formed lesions: after 3 months, miR-31-expressing cells generated only small micrometastases although control cells formed macroscopic metastases; this occurred despite the fact that miR-31-expressing and control cells established comparably sized micrometastases 1 month after injection (Figure 2G; Figure S6B). Such effects on lesion size implied that miR-31 affects metastatic colonization in addition to its influences on local invasion and early postintravasation events.

Inhibition of miR-31 Promotes Metastasis-Relevant Traits In Vitro

The preceding observations demonstrated that miR-31 expression deprives metastatic cells of attributes associated with high-grade malignancy. We next asked whether miR-31 also prevents the acquisition of aggressive traits by otherwise-nonmetastatic human breast cancer cells. To do so, we transiently inhibited miR-31 in noninvasive MCF7-Ras cells with either antisense oligonucleotides or miRNA sponges. The latter are expression constructs that carry miRNA recognition motifs in their 3′ UTR that bind and thus titer miRNAs (Ebert et al., 2007). Both approaches inhibited miR-31 function by >4.5-fold (Figure S7A). Suppression of miR-31 enhanced invasion by 20-fold and motility by 5-fold, but cell viability was unaffected by either inhibitor (Figure 3A; Figure S7B).

Techniques for stable miRNA inhibition have been unavailable (Krützfeldt et al., 2006). To address this problem, we modified elements derived from the transiently expressed miRNA sponges, cloned them into a retroviral vector, and created MCF7-Ras cells that stably express the modified miRNA sponges. The miR-31 sponge reduced miR-31 function by 2.5-fold, but did not affect the activity of other known antimetastatic miRNAs (Figures S8A and S8B). The relatively modest suppression of miR-31 conferred by stable sponge expression elicited strong responses: invasion was enhanced by 12-fold, motility by 8-fold, and anoikis resistance by 2.5-fold (Figure 3B; Figure S8C). The miR-31 sponge failed to alter in vitro proliferation (Figure S8D).

When stably expressed in immortalized HMECs or tumorigenic but nonmetastatic SUM-149 human breast cancer cells, the miR-31 sponge elicited increased invasion, motility, and anoikis resistance without affecting proliferation (Figure S9 and data not shown). Collectively, these data indicated that sustained miR-31 activity is necessary to prevent the acquisition of aggressive traits by both tumor cells and untransformed breast epithelial cells.

Inhibition of miR-31 Promotes Metastasis In Vivo

We exploited our ability to stably inhibit miRNAs in order to assess whether miR-31 activity is required to prevent metastasis in vivo. To do so, otherwise-nonmetastatic MCF7-Ras cells stably expressing the miR-31 sponge were orthotopically implanted into mice. Inhibition of miR-31 failed to alter in vivo proliferation and primary tumor growth (Figure 3C; Figure S10A). Primary tumors derived from miR-31 sponge-expressing cells were poorly encapsulated and locally invasive, whereas control MCF7-Ras tumors appeared well confined and noninvasive (Figures 3D and 3E). Again, neovascularization did not differ (Figure S10B).

Strikingly, miR-31 sponge-expressing MCF7-Ras cells metastasized to the lungs in significant numbers, whereas control tumor-bearing host lungs were largely devoid of tumor cells; cells with impaired miR-31 activity formed 10-fold more lesions than did controls (Figure 3F). Hence, continuous miR-31 function is required to prevent metastasis from an orthotopic site.

We asked whether loss of miR-31 activity also promoted metastasis by intervening at steps of the invasion-metastasis cascade subsequent to local invasion. Thus, we intravenously injected mice with miR-31 sponge-expressing MCF7-Ras cells. Within 1 day, miR-31 inhibition enhanced cell number in the lungs by 6-fold; similarly, at later times after injection, miR-31 sponge-expressing cells were 10-fold more prevalent in the lungs than were controls (Figure 3G). The differing metastatic abilities of control and miR-31 sponge-expressing cells did not arise because of failure of control cells to become lodged initially in the lung vasculature, as shown by the fact that equal numbers of cells from each cohort were present 10 min after injection (Figure 3G; Figure S11).

Suppression of miR-31 also affected lesion size 4 months after tail vein injection: whereas control cells formed only small micrometastases, miR-31 sponge-expressing cells produced macroscopic metastases (Figure 3G). Together, these data extended and reinforced our ectopic expression studies by demonstrating that miR-31 affects local invasion, early postintravasation events, and metastatic colonization.

miR-31 Directly Regulates a Cohort of Prometastatic Genes

miR-31's ability to impede multiple steps of the invasion-metastasis cascade might derive from its ability to pleiotropically regulate genes involved in diverse aspects of metastatic dissemination. To identify effectors of miR-31, we used two algorithms that predict the mRNA targets of a miRNA—PicTar (Krek et al., 2005) and TargetScan (Grimson et al., 2007). Based on the representation of miR-31 sites in their 3′ UTRs, >200 mRNAs were predicted to be regulated by miR-31. Gene ontology (Ashburner et al., 2000) revealed that these targets included a disproportionately large number of genes encoding proteins with roles in motility-related processes, such as cell adhesion, cytoskeletal remodeling, and cell polarity (data not shown).

Guided by this gene ontology analysis, we cloned the 3′ UTRs of 16 putative miR-31 targets from these overrepresented categories, including several implicated in tumor invasion (Sahai and Marshall, 2002; McClatchey, 2003), into a luciferase construct. Reporter assays with miR-31-expressing 231 cells revealed that miR-31 repressed six of the UTRs: frizzled3 (Fzd3), integrin α5 (ITGA5), myosin phosphatase-Rho interacting protein (M-RIP), matrix metallopeptidase 16 (MMP16), radixin (RDX), and RhoA (Figure 4A). Mutation of the putative miR-31 site(s) in these six 3′ UTRs (Table S3) abrogated responsiveness to miR-31 (Figure 4B). In the case of RhoA, whose UTR contains two miR-31 sites separated by 152 nucleotides, mutation of either motif abolished miR-31 responsiveness (Figure 4B), suggesting functional interaction between the sites (Grimson et al., 2007).

Endogenous Fzd3, ITGA5, MMP16, RDX, and RhoA protein levels were assayed in miR-31-expressing 231 cells. miR-31 repressed the levels of these proteins by 40%–60% (Figure 4C). miR-31's effects on levels of the M-RIP protein could not be evaluated because of the lack of appropriate antibodies. Also, miR-31 reduced the endogenous mRNA levels of these six targets by 2-fold in SUM-159 cells, as well as Fzd3, ITGA5, MMP16, RDX, and RhoA mRNA levels in 231 cells (Figure 4D). miR-31 did not affect CXCL12 mRNA levels—a computationally predicted miR-31 target found not to be regulated by this miRNA—in either cell type (Figures 4A and 4D). These data indicated that miR-31 directly regulates endogenous Fzd3, ITGA5,

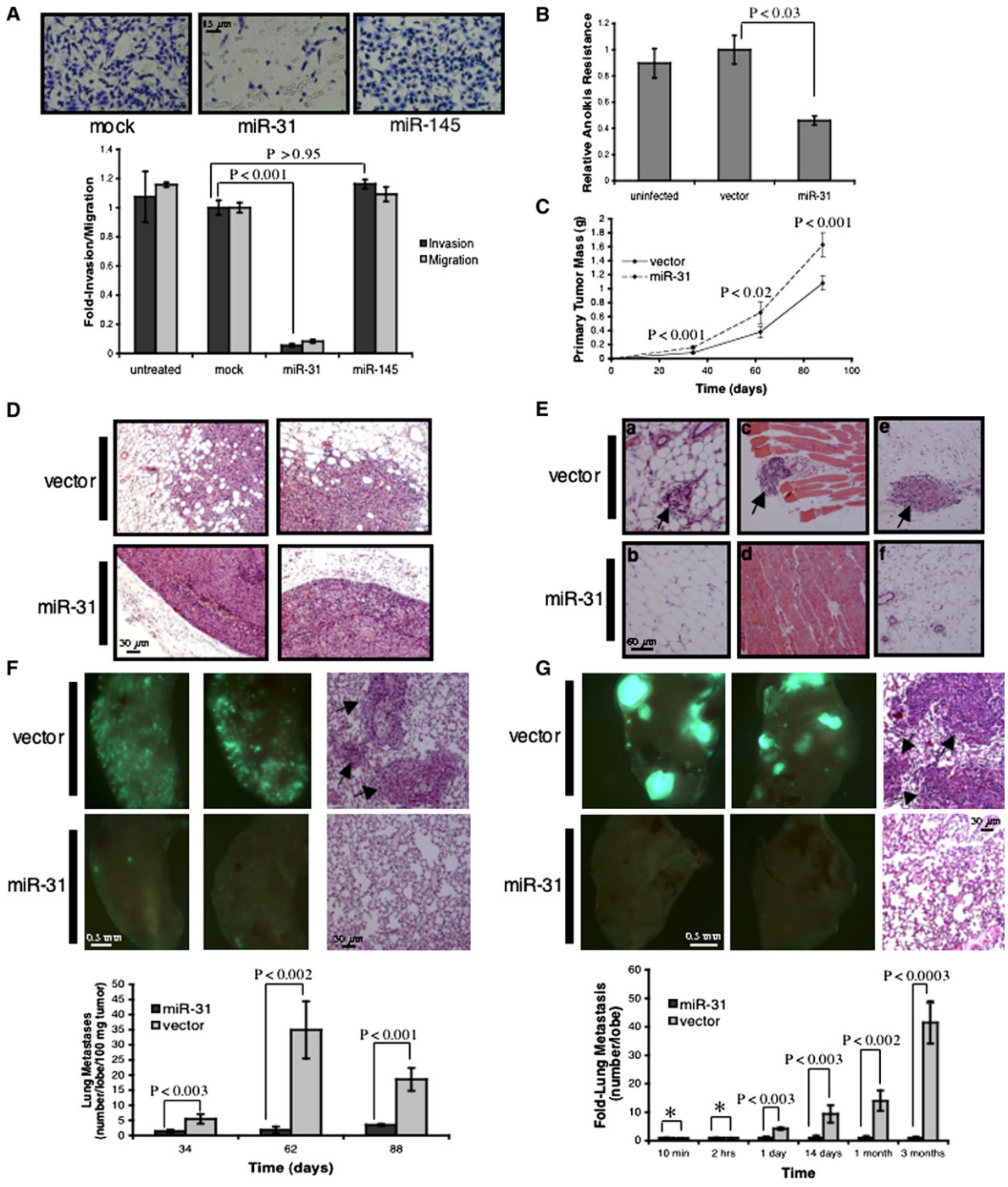

Figure 2. miR-31 Expression Inhibits Metastasis

(A) Invasion and motility assays after transfection of MDA-MB-231 (231) cells with the indicated constructs. n = 3.

(B) Anoikis assays with 231 cells infected as indicated. n = 3.

(C) Primary tumor growth upon orthotopic injection of 1.0 × 10^6 GFP-labeled 231 cells infected as indicated. The experiment was terminated after 13 weeks because of primary tumor burden. n = 5 per group per time point.

(D) H&E stain of 231 primary tumors 62 days after orthotopic injection.

(E) H&E stain of tissue adjacent to the indicated 231 primary mammary tumors 62 days after injection. Arrows: disseminated tumor cells in normal fat (a, b), muscle (c, d), and subcutis (e, f).

(F) Images of murine lungs to visualize GFP-labeled 231 cells 62 days after orthotopic implantation (left). H&E stain of lungs from animals bearing the indicated tumors (right); arrows indicate metastatic foci. n = 5.

M-RIP, MMP16, RDX, and RhoA expression in human breast cancer cells.

We determined whether concomitant repression of Fzd3, ITGA5, M-RIP, MMP16, RDX, and RhoA correlated with disease progression in clinical breast cancers by examining expression profiling data from 295 primary breast tumors (Table S4; van de Vijver et al., 2002). To do so, we constructed a miR-31 target signature based on coordinate differential expression of these six genes. Within this cohort, high expression of the miR-31 target signature was associated with metastasis, as well as poor survival, relative to signature-negative tumors; 5-year survival among patients negative for the target signature was 90%, whereas >35% of target signature-positive patients succumbed to their disease over this interval (Figures 5A and 5B). Thus, coordinate repression of Fzd3, ITGA5, M-RIP, MMP16, RDX, and RhoA correlated with more favorable outcome in clinical breast tumors.

To assess the functional contributions of these miR-31 targets to aggressive phenotypes, we first examined whether their inhibition affected the invasion or motility of 231 cells. Transfection with siRNAs potently reduced target protein levels without affecting cell viability (Figures S12A and S12B). siRNAs targeting Fzd3, ITGA5, RDX, or RhoA reduced invasion and motility, whereas siRNAs against M-RIP or MMP16 failed to affect these traits (Figure 5C; Figure S12C).

We asked whether inhibition of these effectors compromised resistance to anoikis. siRNAs against ITGA5, RDX, or RhoA sensitized 231 cells to anoikis; in contrast, siRNAs targeting Fzd3, M-RIP, or MMP16 had no effect on anoikis resistance (Figure 5D). Hence, suppression of Fzd3, ITGA5, RDX, or RhoA impaired metastasis-relevant traits in vitro.

Re-expression of Fzd3, ITGA5, RDX, and RhoA Reverses miR-31-Dependent Metastasis-Relevant Phenotypes In Vitro

To determine whether in vitro phenotypes associated with miR-31 expression could be reversed via restoration of Fzd3, ITGA5, M-RIP, MMP16, RDX, or RhoA levels, we transfected miR-31-expressing 231 cells with individual expression constructs rendered miRNA insensitive by deletion of their 3′ UTRs; this was not cytotoxic (Figures S13A and S13B and data not shown). In miR-31-expressing cells, Fzd3, ITGA5, RDX, or RhoA reversed, at least partially, miR-31-imposed invasion and motility defects; in contrast, M-RIP or MMP16 had no effect on these traits (Figure 5E; Figure S13C). Surprisingly, re-expression of RDX or RhoA completely rescued miR-31-mediated invasion and motility defects. Expression of the six targets failed to enhance the invasion or motility of control 231 cells (Figure 5E; Figure S13C).

We evaluated whether re-expression of any of the six targets rescued miR-31's effects on anoikis. ITGA5, RDX, or RhoA reversed, at least in part, anoikis susceptibility resulting from ectopic miR-31; in contrast, Fzd3, M-RIP, or MMP16 failed to affect this trait (Figure 5F). In fact, ITGA5 or RhoA completely rescued miR-31-dependent anoikis phenotypes. The six targets did not enhance anoikis resistance in control 231 cells (Figure 5F). Hence, Fzd3, ITGA5, RDX, and RhoA are functionally relevant effectors of miR-31 for conferring malignant traits in vitro.

Re-expression of RhoA Partially Reverses miR-31-Imposed Metastasis Defects In Vivo

RhoA afforded the most pronounced reversal of miR-31-mediated phenotypes. Therefore, we stably re-expressed miRNA-resistant RhoA in 231 cells that already had been infected with either miR-31 or control vector (Figures S14A and S14B). RhoA did not affect proliferation in vitro, but did abrogate miR-31-imposed invasion, motility, and anoikis resistance defects (Figures S14C–S14F).

To ascertain whether restored RhoA levels reversed in vivo metastasis phenotypes ascribable to miR-31, we orthotopically injected mice with 231 cells expressing combinations of miR-31, RhoA, and control vectors. As observed previously, miR-31 enhanced primary tumor growth (Figure 6A). RhoA initially augmented primary tumor growth in the presence of ectopic miR-31, but failed to do so in control 231 cells (Figure 6A). In consonance with our earlier findings, control 231 primary tumors were locally invasive and miR-31-expressing tumors were noninvasive (Figures 6B and 6C). In control 231 cells, ectopic RhoA failed to exacerbate the extent of local invasion; in contrast, RhoA abolished the previously encapsulated appearance of miR-31-expressing tumors and enabled invasion into surrounding normal tissue (Figures 6B and 6C).

Re-expression of RhoA restored lung metastasis in miR-31-expressing 231 cells to 75% of control cell levels, although RhoA failed to enhance metastasis in control 231 cells (Figure 6D). Thus, re-expression of RhoA partially, yet robustly, reverses metastasis suppression imposed by miR-31. The observed magnitude of rescue is surprising, because RhoA is only one member of a larger cohort of metastasis-relevant genes repressed by miR-31.

By intravenously injecting mice with 231 cells expressing miR-31 and/or RhoA, we gauged whether RhoA-mediated reversal of miR-31-imposed metastasis defects was solely attributable to effects on local invasion. Although expression of miR-31 and/or RhoA failed to affect the initial lodging of tumor cells in the lung vasculature, the number of cells that persisted in the lungs differed within one day of injection (Figure 6E; Figure S15). As before, miR-31 inhibited both the number of metastases formed and their eventual size (Figure 6E). Although expression of RhoA in control 231 cells failed to enhance metastasis, RhoA restored the number of lung metastases to 60% of control cell levels in miR-31-expressing cells; however, RhoA did not facilitate the formation of macroscopic metastases in cells with ectopic miR-31 (Figure 6E).

Together, these data indicated that miR-31's ability to inhibit metastasis is attributable, in significant part, to its capacity to inhibit RhoA. miR-31-mediated repression of RhoA affects

(G) Images of murine lungs to detect GFP-labeled 231 cells 88 days after tail vein injection (left). H&E stain of lungs (right); arrows indicate metastatic foci. Asterisks: $p > 0.66$. $n = 5$, except for 10 min and 2 hr ($n = 4$).
Data are presented as mean ± SEM.

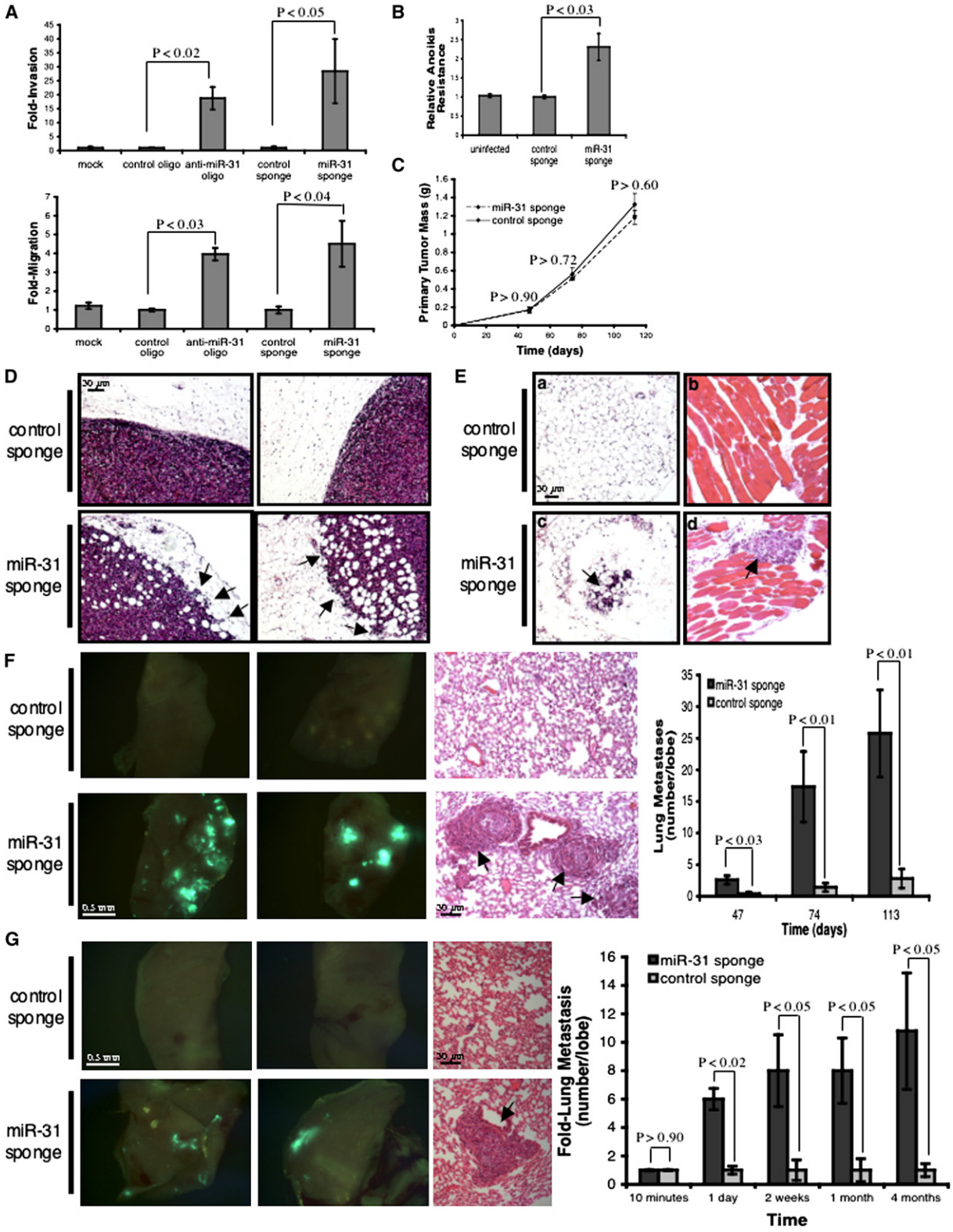

Figure 3. Inhibition of miR-31 Promotes Metastasis

(A) Invasion and motility assays with MCF7-Ras cells transfected with the indicated transient miR-31 inhibitors. n = 3.

(B) Anoikis assays with MCF7-Ras cells stably expressing the indicated sponge. n = 3.

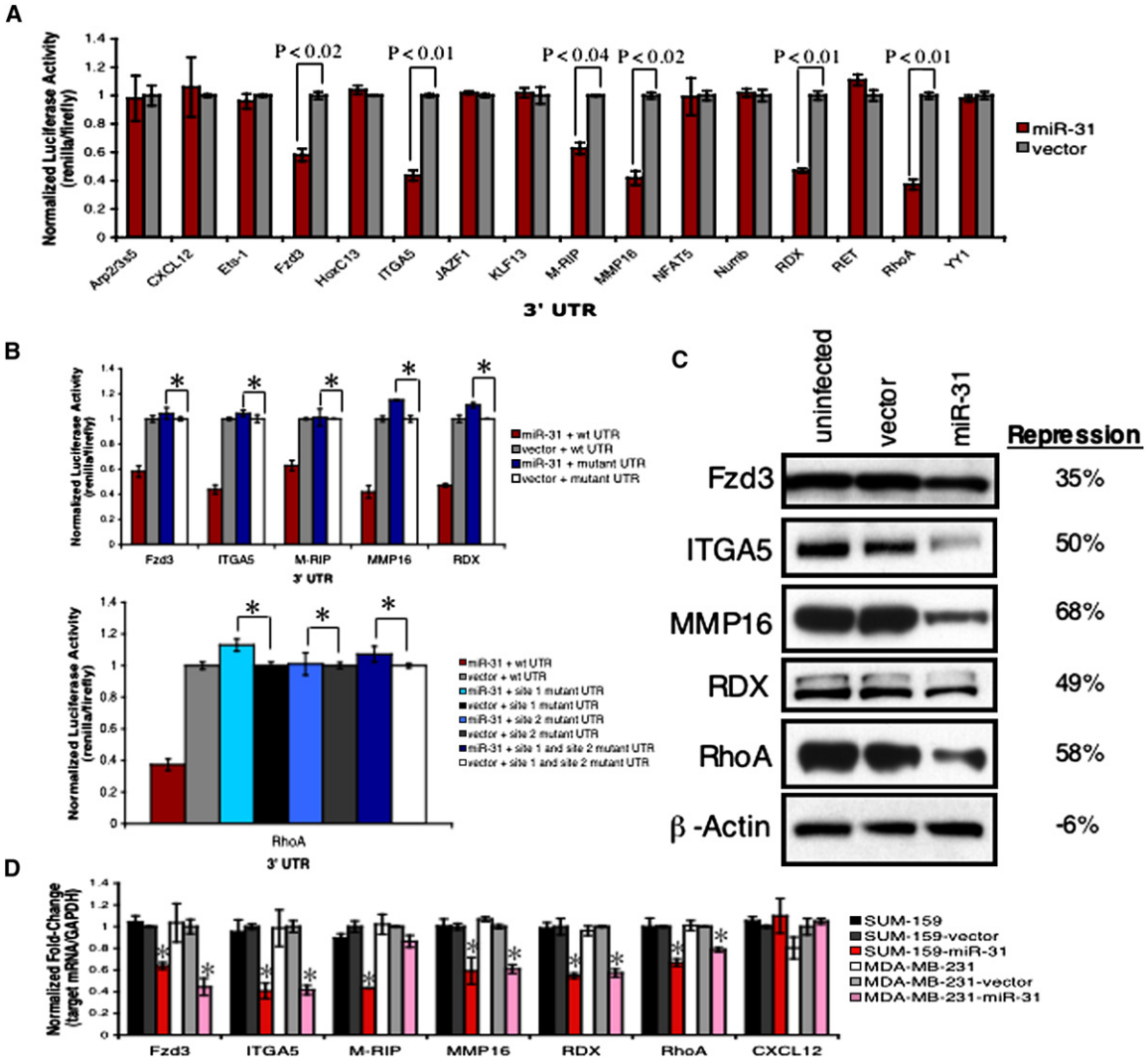

Figure 4. miR-31 Directly Regulates a Cohort of Prometastatic Genes

(A) Luciferase activity in 231 cells infected with miR-31 or control vector after transfection of the indicated 3′ UTR-driven reporter constructs. n = 3.

(B) Luciferase activity in the indicated 231 cells upon transfection of miR-31 site mutant 3′ UTR-driven reporter constructs. wt: wild-type; site 1: the miR-31 motif at nt 145–151 of the RhoA 3′ UTR; site 2: the motif spanning nt 303–309. Asterisks: $p > 0.80$ relative to mutant-UTR + vector controls. n = 3.

(C) Immunoblots for endogenous Fzd3, ITGA5, MMP16, RDX, and RhoA in the indicated 231 cells. β-actin was a loading control. Repression: protein levels in miR-31-expressing cells relative to vector controls.

(D) RT-PCR for endogenous CXCL12, Fzd3, ITGA5, M-RIP, MMP16, RDX, and RhoA. GAPDH was a loading control. Asterisks: $p < 0.03$ relative to vector controls. n = 3.

Data are presented as mean ± SEM.

both local invasion and early postintravasation events. However, these data also implied that the full spectrum of miR-31's effects on metastasis are elicited only via the coordinate repression of multiple targets, because suppression of RhoA alone could not explain the complete impact of miR-31 on the number of metastases formed or its effects on metastatic colonization.

(C) Primary tumor growth upon orthotopic implantation of 5.0×10^5 GFP-labeled MCF7-Ras cells infected as indicated. The experiment was terminated after 16 weeks because of primary tumor burden. n = 5 per group per time point.

(D) H&E stain of MCF7-Ras primary tumors 47 days after orthotopic injection. Arrows indicate regions of poor encapsulation.

(E) H&E stain of tissue adjacent to the indicated MCF7-Ras primary tumors 47 days postinjection. Arrows: disseminated tumor cells in normal fat (a, c) and muscle (b, d).

(F) Images of murine lungs to visualize GFP-labeled MCF7-Ras cells 113 days after orthotopic injection (left). H&E stain of lungs from animals bearing the indicated tumors (middle); arrows indicate metastatic foci. n = 5.

(G) Images of murine lungs to detect GFP-labeled MCF7-Ras cells 122 days after tail vein injection (left). H&E stain of lungs (middle); arrow indicates metastasis. n = 4, except for 1 day (n = 3).

Data are presented as mean ± SEM.

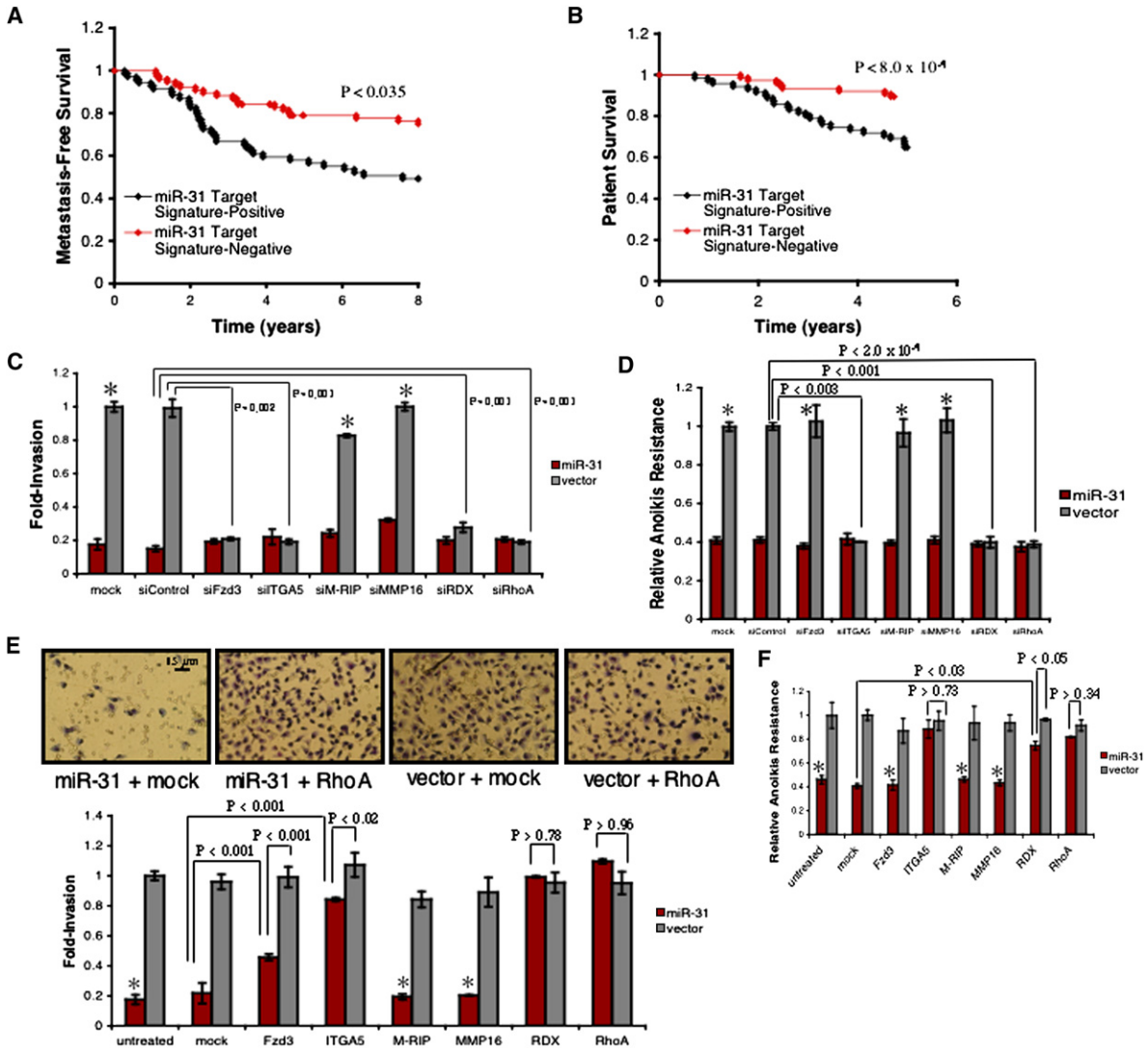

Figure 5. Repression of Fzd3, ITGA5, RDX, and RhoA Underlies miR-31-Dependent Phenotypes In Vitro

(A) Kaplan-Meier curves for 295 human primary breast tumors depicting metastasis-free survival, stratified based on expression of the 6-gene miR-31 target signature. p value based on a logrank test.

(B) Kaplan-Meier 5-year survival curves for 295 breast cancer patients, stratified based on miR-31 target signature expression in their primary tumors. p value based on a logrank test.

(C) Invasion assays with miR-31-expressing or control 231 cells transfected as indicated. Asterisks: $p > 0.19$ relative to vector + siControl cells. n = 3.

(D) Anoikis assays with 231 cells transfected with the indicated siRNAs. Asterisks: $p > 0.80$ relative to vector + siControl cells. n = 3.

(E) Invasion assays with the indicated 231 cells transfected with miRNA-resistant expression constructs. Asterisks: $p > 0.61$ relative to miR-31 + mock cells. n = 3.

(F) Anoikis assays with the indicated 231 cells transfected as noted. Asterisks: $p > 0.11$ relative to miR-31 + mock cells. n = 3.

Data are presented as mean ± SEM.

miR-31 Expression Correlates Inversely with Metastasis in Human Breast Tumors

Because established cell lines and xenograft studies cannot fully recapitulate clinical malignancy, we extended our analyses by assaying miR-31 expression in specimens from 56 human breast cancer patients (Table S5; median follow-up = 59 months). Relative to grade-matched estrogen receptor $(ER)^+$ tumors, which are associated with more favorable disease outcome (Sørlie et al., 2001), basal-like tumors exhibited 40% reduced miR-31; no difference in miR-31 levels was observed between ER^+ and $HER2^+$ tumors (Figure S16).

When these 56 tumors were stratified based on clinical progression, we found that miR-31 expression was diminished in primary tumors that subsequently metastasized, when compared to normal breast tissue and primary tumors that did not recur; moreover, low miR-31 levels correlated strongly with reduced distant disease-free survival relative to tumors with high miR-31 (Figures 7A and 7B). Similarly, within this cohort of

tumors, high RhoA expression was associated with an increased incidence of distant metastasis (Figure S17).

The association of low miR-31 levels with metastasis persisted independent of both tumor grade and molecular subtype (Figure S18). Such grade and subtype independence is quite surprising, because clinically utilized prognostic markers for breast cancer largely correlate with these parameters; furthermore, currently available markers do not identify a worse-prognosis group within the more aggressive basal-like or $HER2^+$ subtypes (Desmedt et al., 2008). Thus, miR-31 may represent a marker for metastasis in a variety of breast cancer subtypes; however, its utility as a prognostic indicator will depend on extension of these initial observations.

We next assessed the heterogeneity of miR-31 expression in human primary breast tumors, as well as distant metastases arising in the same patients. miR-31 was expressed in 65% of the cells in these primary tumors; however, miR-31 was detected in only 12%–30% of cells in patient-matched distant metastases (Figure 7C). These data raise the possibility that selective pressures operating over the course of breast cancer progression diminish the representation of miR-31-expressing cells within the population of successfully metastasizing cells.

Finally, we asked whether expression of ITGA5, RDX, and RhoA was also heterogeneous in primary human breast tumors. RDX and RhoA were expressed in 60%–75% of cells in the primary tumors examined, whereas ITGA5 was detected in >80% of cells (Figure 7D). Distant metastases were more homogeneous for the expression of RDX and RhoA than the primary tumors from which they were derived, as indicated by the fact that >90% of cells in the metastases expressed RDX and RhoA (Figure 7D). Similarly, >90% of cells in the metastases expressed ITGA5; however, the widespread ITGA5 expression observed in the patient-matched primary tumors complicates interpretation of its expression in distant metastases (Figure 7D).

DISCUSSION

miRNAs can modulate a wide variety of biological processes. In the present report, we demonstrate that a single human miRNA, miR-31, is endowed with the ability to concomitantly repress multiple prometastatic targets and to thereby inhibit several distinct steps of the invasion-metastasis cascade. Moreover, miR-31 levels correlate inversely with metastatic recurrence in a cohort of human breast tumors, a preliminary association that appears to persist independent of both tumor grade and subtype.

Genome-wide studies have described miR-31 downregulation or deletion of the miR-31 genomic locus in human breast cancers (Calin et al., 2004; Zhang et al., 2006; Yan et al., 2008). Expression profiling of clinical breast tumors revealed reduced miR-31 in luminal B (relative to luminal A), basal-like, and $HER2^+$ tumors (Mattie et al., 2006; Blenkiron et al., 2007)—patterns of reduction that correlate with aggressive disease (Sørlie et al., 2001). In contrast, another profiling study found elevated miR-31 in human breast tumors (Volinia et al., 2006). None of these studies stratified patients by metastasis status.

A limited number of miRNAs with prometastic (miR-10b, -21, and -373/520c) or antimetastatic (miR-34b/c, -126, -148a, -206, and -335) functions have been identified. However, the contributions of miR-10b, miR-21, and miR-373/520c specifically to metastasis promotion are not easily discerned because of their mitogenic and/or antiapoptotic roles (Voorhoeve et al., 2006; Ma et al., 2007; Si et al., 2007). Similarly, the antimetastatic miRNAs miR-34b/c, miR-126, and miR-148a impair primary tumor growth (Lujambio et al., 2008; Tavazoie et al., 2008), whereas miR-206 and miR-335 inhibit proliferation or promote apoptosis (Sathyan et al., 2007; Kondo et al., 2008), again obscuring their precise roles in metastasis.

In contrast, miR-31 obstructs metastasis without exerting confounding influences on primary tumor development. As such, *mir-31* might aptly be categorized as a "metastasis suppressor gene" (Steeg, 2003). This unique aspect of miR-31 function, among others, raises questions regarding the still-uncharacterized role of this miRNA in normal cell and organismic physiology. Of significance, loss of miR-31 activity enhances invasiveness, motility, and anoikis resistance in untransformed human mammary epithelial cells. Hence, inactivation of miR-31 in normal epithelium may facilitate dissemination prior to transformation to a fully neoplastic state. This suggests one putative mechanism by which the invasion-metastasis cascade could be initiated very early during the course of tumor progression, a phenomenon that has recently been observed in clinical breast tumors (Hüsemann et al., 2008).

Given the capacity of miR-31 to enhance primary tumor growth, an oncogenic role for this miRNA (mechanistically independent of its metastasis-suppressive functions) cannot be formally excluded. Such duality of action is not unprecedented (Massagué, 2008) and is consistent with notions that metastasis- and tumorigenesis-enabling attributes can be biologically distinct and acquired via independent selective pressures during malignant progression.

Previous studies have described effects of specific miRNAs on an early stage of the invasion-metastasis cascade—local invasion. The present work demonstrates that miRNAs can also influence later steps of metastasis and that an individual miRNA can intervene at multiple distinct stages of the invasion-metastasis cascade. miR-31 regulates the local invasion of primary mammary tumors, as well as intraluminal survival, extravasation, and/or initial viability in a foreign microenvironment. miR-31 also suppresses colonization—the final and rate-limiting step of metastasis (Fidler, 2003). miR-31-imposed suppression of RhoA partially explains the effects of this miRNA on local invasion and early postintravasation events; however, the mechanisms by which miR-31 suppresses metastatic colonization remain unresolved.

The levels of several functionally relevant effectors of miR-31 correlate with disease progression in human tumors. RhoA expression, for example, is elevated in aggressive breast neoplasias (Sahai and Marshall, 2002). Similar associations have been described in human tumors for ITGA5 (Sanchez-Carbayo et al., 2006) and the RDX family (McClatchey, 2003).

Re-expression of several individual miR-31 targets largely reversed miR-31-imposed defects in vitro. This may indicate that certain miR-31 effectors activate one another; however, ectopic ITGA5, RDX, or RhoA did not induce the expression of other miR-31 targets (data not shown). Alternatively, available

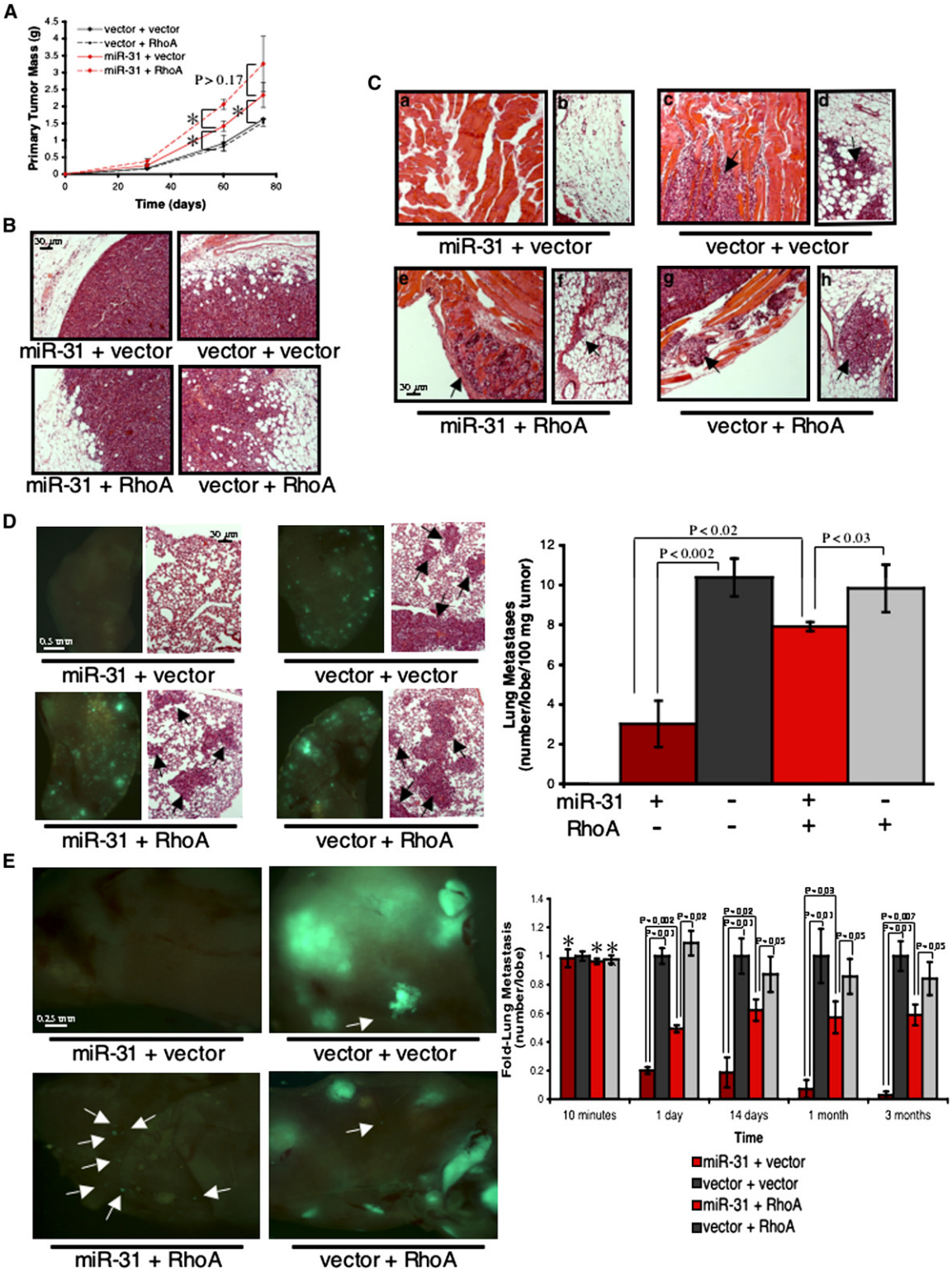

Figure 6. Re-expression of RhoA Partially Reverses miR-31-Imposed Metastasis Defects In Vivo

(A) Primary tumor growth upon orthotopic injection of 5.0×10^5 GFP-labeled 231 cells. The experiment was terminated after 11 weeks because of primary tumor burden. Asterisks: $p < 0.02$. n = 5 per group per time point.

(B) H&E stain of 231 primary tumors 60 days after orthotopic injection.

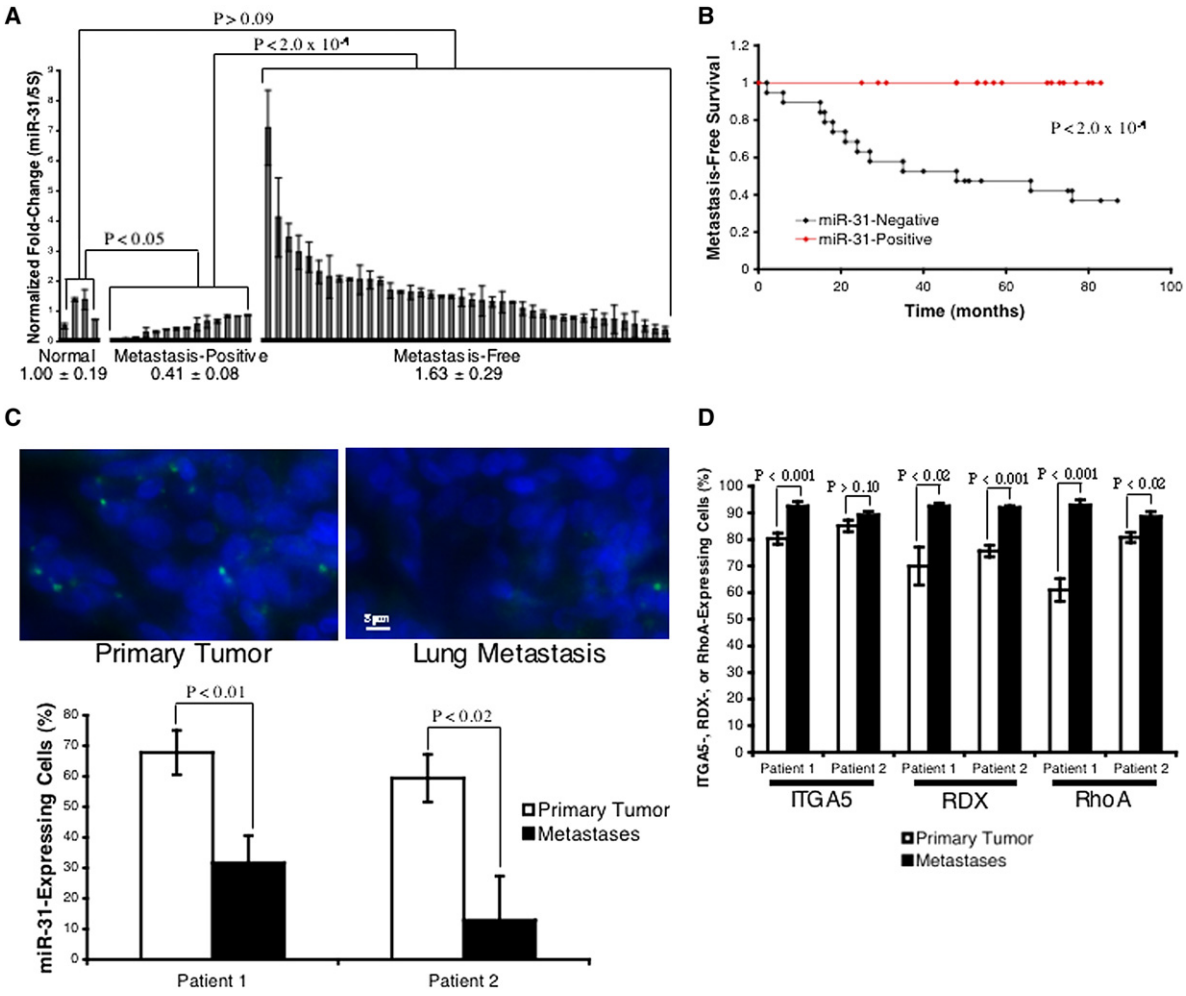

Figure 7. miR-31 Levels Correlate Inversely with Metastasis in Human Breast Tumors

(A) miR-31 RT-PCR in 54 primary breast tumors. Normal: tissue from nondiseased individuals; metastasis-positive and -free: tumors of the indicated distant metastasis outcome. 5S rRNA was a loading control. n = 4 (normal); n = 14 (metastasis-positive); n = 40 (metastasis-free).

(B) Kaplan-Meier distant metastasis-free survival curves for 54 breast cancer patients, stratified based on miR-31 levels in their primary tumors. p value based on a chi-square test.

(C) In situ hybridization for miR-31 (green) in patient-matched primary breast tumors and distant metastases (patient 1 = lung; 2 = pleura); DAPI counterstain (blue). n = 8 fields.

(D) Immunohistochemical detection of ITGA5, RDX, and RhoA in patient-matched primary breast tumors and distant metastases (patient 1 = lung; 2 = pleura). n = 8 fields.

Data are presented as mean ± SEM.

in vitro assays might inadequately model the complexity of metastasis; hence, in vivo manifestations of modeled behaviors may require the concurrent action of multiple miR-31 effectors. Also, not all steps of metastasis can be recapitulated in vitro. Consistent with these notions, RhoA completely reversed a number of miR-31-dependent defects in vitro, yet only partially rescued miR-31-imposed metastasis phenotypes in vivo. This supports beliefs that miRNAs act via the pleiotropic regulation of multiple effectors.

Our analyses rely on established human cell lines and xenograft studies, approaches that cannot fully simulate clinical carcinomas. For example, cell lines accumulate genetic changes

(C) H&E stain of tissue adjacent to the indicated 231 primary mammary tumors 60 days after injection. Arrows indicate disseminated tumor cells in normal muscle (a, c, e, g) and fat (b, d, f, h).

(D) Images of murine lungs to visualize GFP-labeled 231 cells 60 days after orthotopic injection (left). H&E stain of lungs from animals bearing the indicated tumors (right); arrows indicate metastatic foci. n = 5.

(E) Images of murine lungs to detect GFP-labeled 231 cells 86 days after tail vein injection (left); arrows indicate micrometastatic lesions. Asterisks: $p > 0.87$ relative to vector + vector controls. n = 4, except for 2 weeks (n = 3).

Data are presented as mean ± SEM.

in culture, whereas xenografts fail to recapitulate species-specific interactions between tumor cells and their stroma. However, the consistency of our results upon use of multiple independent cell lines (including a single-cell-derived population), the convergence of our gain- and loss-of-function findings, and our correlative studies in human breast cancer patients and murine mammary tumor cell lines argue against major confounding influences stemming from our experimental models.

Collectively, the findings of the present study carry significant implications regarding our understanding of the pathogenesis of high-grade malignancies. Our data suggest that the loss of a single gene product can facilitate the completion of multiple distinct steps of the invasion-metastasis cascade; this pleiotropic action may help to explain how tumor cells can accumulate enough genetic and epigenetic aberrations over the course of a human lifespan to overcome the numerous barriers that normally operate to prevent metastasis. Moreover, because distant metastases are responsible for patient mortality in the vast majority of human carcinomas, miR-31's ability to impede metastasis may prove to be clinically useful.

EXPERIMENTAL PROCEDURES

Cell Culture

MDA-MB-231 and MCF7-Ras cells were obtained from the ATCC and cultured under standard conditions. HMEC and HME cells have been described (Ma et al., 2007). SCP3 cells were obtained from J. Massagué (Minn et al., 2005). SUM-149 and -159 cells were provided by S. Ethier (Ma et al., 2007). D2 cells have been described (Morris et al., 1993). 67NR, 168FARN, 4TO7, and 4T1 cells were obtained from F. Miller (Aslakson and Miller, 1992).

miRNA Detection

Total RNA, inclusive of the small RNA fraction, was extracted from cultured cells with a *mir*Vana miRNA Isolation Kit (Ambion). RT-PCR-based detection of mature miR-31 and 5S rRNA was achieved with a *mir*Vana miRNA Detection Kit and gene-specific primers (Ambion).

miRNA In Situ Hybridization

miRNA expression was assessed from paraffin sections via a protocol adapted from Silahtaroglu et al., (2007). In brief, after a 4 hr prehybridization, a 5′ FITC-labeled miRCURY LNA probe targeting miR-31 (Exiqon) was hybridized to proteinase K-treated 10 μm sections at 55°C for 12 hr. Slides were then incubated with anti-FITC-HRP (PerkinElmer), and the resulting signal was intensified with the TSA Plus Fluorescein System (PerkinElmer).

Human Breast Tumors

Primary breast tumors, distant metastases, and normal breast tissue were collected and processed in compliance with a protocol approved by the Brigham and Women's Hospital IRB. Fresh tissue was harvested from patients, OCT-embedded, snap-frozen, and preserved at −80°C. Recurrent cases were primary tumors from patients that developed distant metastases. For each recurrent case, two nonrecurrent cases were selected to control for date of diagnosis, molecular subtype, lymph node status, and time of follow-up. Total RNA was isolated from 35 μm sections via TRIzol extraction and a *mir*Vana miRNA Isolation Kit. To discern whether miR-31 levels correlate with distant metastasis, primary tumors were classified as miR-31 positive or negative. Tumors were considered miR-31 positive or negative if the normalized expression of miR-31 resided in the top or bottom 30% of tumors in this cohort, respectively. Similarly, tumors were classified as RhoA high or low if their RhoA levels were in the top or bottom 30% of tumors examined.

Invasion and Motility Assays

For invasion assays, 1.0×10^5 cells were seeded in a Matrigel-coated chamber with 8.0 μm pores (BD Biosciences); for motility assays, 5.0×10^4 cells were plated atop uncoated membranes with 8.0 μm pores (BD Biosciences). Cells were seeded in serum-free media and translocated toward complete growth media for 20 hr. Fugene6 (Roche) was used to transfect cells 24 hr prior to plating. 200 nM miRIDIAN miRNA Inhibitors (Dharmacon) were employed to transiently inhibit miR-31. SMARTpool siRNAs against Fzd3, ITGA5, M-RIP, MMP16, RDX, or RhoA (Dharmacon) were provided at 100 nM. Antisense oligonucleotides and siRNAs were transfected 48 hr prior to seeding with Oligofectamine (Invitrogen).

Anoikis Assays

Anoikis resistance was evaluated by seeding 7.5×10^4 cells in ultralow attachment plates (Corning). After 24 hr of anchorage-independent culture, cells were resuspended in 0.4% trypan blue (Sigma) and cell viability was assessed.

Animal Studies

All research involving animals complied with protocols approved by the MIT Committee on Animal Care. For spontaneous metastasis assays, age-matched female NOD/SCID mice (propagated on-site) were bilaterally injected into the mammary fat pad with the indicated number of tumor cells in 1:2 Matrigel (BD Biosciences) plus normal growth media. For experimental metastasis assays, age-matched female NOD/SCID mice were injected with 5.0×10^5 cells (resuspended in PBS) via the tail vein. Metastasis was quantified with a fluorescent microscope within 3 hr of specimen isolation.

Luciferase Assays

5.0×10^4 cells were cotransfected with 50 ng of the indicated pIS1 *Renilla* luciferase construct and 50 ng of a pIS0 firefly luciferase normalization control. Lysates were collected 24 hr after transfection, and *Renilla* and firefly luciferase activities were measured with a Dual-Luciferase Reporter System (Promega).

Immunoblots

Lysates were resolved by electrophoresis, transferred to a PVDF membrane, and probed with antibodies against β-actin (Santa Cruz), Fzd3 (Abcam), ITGA5 (Santa Cruz), MMP16 (Abcam), RDX (Cell Signaling), or RhoA (Santa Cruz).

miR-31 Target Signature

Expression profiling of 295 human breast tumors (van de Vijver et al., 2002) was used to categorize tumors as miR-31 target signature positive or negative. Tumors were considered target signature positive or negative if the normalized expression of multiple of the six miR-31 targets herein identified resided in the top or bottom 15% of tumors in this cohort, respectively.

Immunohistochemistry

Detection of Ki-67 (PharMingen), MECA-32 (U. of Iowa), ITGA5 (Santa Cruz), RDX (Santa Cruz), or RhoA (Abcam) was performed on 5 μm paraffin sections with the indicated antibodies, Vectastain Elite ABC kits (Vector), and ImmPACT DAB Substrate (Vector).

Statistical Analyses

Data are presented as mean ± SEM. Unless otherwise noted, Student's t test was used for comparisons, with $p < 0.05$ considered significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 18 figures, and 5 tables and can be found with this article online at http://www.cell.com/cell/supplemental/S0092-8674(09)00390-0.

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MicroRNAs: Crucial multi-tasking components in the complex circuitry of tumor metastasis

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Distant metastases are the underlying cause of patient mortality in an overwhelming majority of human carcinomas. Certain microRNAs have recently been found capable of regulating the process of tumor metastasis. In this review, we highlight advances within this rapidly emerging field, endeavor to connect known microRNA pathways with recent conceptual advances in the larger field of metastasis research, and speculate regarding the future utility of microRNAs in the diagnosis and treatment of human cancers. Assessed collectively, current evidence suggests that the pleiotropic activities of microRNAs endow them with the capacity to function as crucial, yet previously unappreciated, nodes within already-identified metastasis regulatory circuitry. This has important implications for our understanding of the pathogenesis of high-grade malignancies.

Introduction

Metastases, rather than the primary tumor from which these growths arise, are responsible for greater than 90% of patient mortality from solid tumors.[1] In order to metastasize, cells in a primary tumor must first complete a multi-step program termed the "invasion-metastasis cascade", during which the cancer cells become motile, invade through local extracellular matrix and stroma, intravasate into the lumen of a vessel, survive transport through the vasculature, extravasate into the parenchyma of a distant tissue, survive within this foreign microenvironment, form micrometastases, and re-initiate their proliferative machinery to establish macroscopic secondary tumors via a process termed colonization.[2] Each step of the invasion-metastasis cascade is believed to be accompanied by a significant rate of attrition; hence, the number of carcinoma cells disseminated from a primary tumor greatly exceeds (often by orders of magnitude) the number of metastases that ultimately develop.[3] Despite the vital clinical significance of metastasis for determining disease outcome in human neoplasias, our present understanding of metastasis-regulating signaling networks remains incomplete.[1]

MicroRNAs (miRNAs) were originally identified through forward genetic screens in the nematode *C. elegans*.[4,5] Initially considered a peculiarity of worm development, it was only years later—after the identification of the first miRNA that was evolutionarily conserved from nematodes to humans[6]—that these small non-coding RNAs began to attract widespread attention.[7] The recent, rapidly expanding interest in miRNAs has led to remarkable progress over a relatively short period of time; we are now beginning to understand in significant detail the biogenesis of miRNAs, their targeting specificity, and the mechanisms by which they silence gene expression post-transcriptionally.[8,9]

At present, more than 650 human miRNAs have been identified.[9] As each individual miRNA is capable of modulating the expression of dozens of distinct target mRNAs, current estimates posit that greater than one-third of the mRNA species encoded in the human genome are subject to miRNA-mediated regulation.[9] Moreover, the pleiotropic actions of individual miRNAs enable them to function as crucial regulators of cell and organismal homeostasis; indeed, specific miRNAs critically modulate a variety of normal physiologic processes.[7] Additionally, aberrant miRNA activity contributes to a number of pathological states,[7] including tumor development and metastasis.[10-12]

A role for miRNAs in cancer was first revealed by the work of Croce and colleagues, who discovered that miR-15 and miR-16 were frequently deleted in human chronic lymphocytic leukemias.[13] Subsequent analyses revealed that these two miRNAs function as bona fide tumor suppressor genes by inducing cell cycle arrest and apoptosis.[14] Provocatively, more than 50% of miRNA-encoding genomic loci reside in chromosomal regions that are known to be altered during the course of tumor pathogenesis,[15] and global downregulation of miRNA levels in human tumors has been reported.[16] Furthermore, characteristic miRNA expression profiles that are strongly associated with both disease status and ultimate clinical outcome have been identified in many types of neoplasias.[14] Alterations in miRNA levels occurring during tumor development are not epiphenomena of cancer pathogenesis, since genetic inhibition of the miRNA biogenesis machinery accelerates tumor progression;[17] consequently, modulation of the expression of certain miRNAs is likely to play a causal role in tumorigenesis.

Research conducted over the past five years has revealed that the genes encoding a number of miRNAs behave as classically defined oncogenes or tumor suppressor genes; several recent reviews have summarized these findings, and therefore we will

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not discuss them here.[10,12,18] Instead, this article will summarize our current knowledge regarding the role of miRNAs specifically in metastatic progression. We highlight several recent conceptual advances within the larger field of metastasis research and examine the contributions of miRNAs to the acquisition of these malignant phenotypes. Finally, we speculate about the potential impact of this rapidly expanding knowledge on the diagnosis and treatment of metastatic human cancers.

MicroRNAs as Critical Regulators of Tumor Metastasis

miR-10b was the first miRNA recognized to alter the metastatic potential of human cancer cells.[19] Via gain-of-function approaches, Ma and colleagues demonstrated that ectopic miR-10b expression endowed otherwise-non-aggressive human breast cancer cells with the capacity to become motile and invasive, as well as seed distant micrometastases when implanted as xenografts in immunodeficient mice.[19] Subsequently, an unbiased functional genetic screen involving overexpression of approximately 450 miRNAs by the Agami group identified the miR-373/520c seed family as pro-metastatic miRNAs in human breast cancer cells; once again, these effects were ostensibly due to the ability of these miRNAs to promote cell motility and invasiveness.[20] Soon thereafter, research from two other laboratories highlighted miR-21 as yet another motility- and metastasis-promoting miRNA in human breast and colorectal carcinoma cells.[21,22]

By combining in vivo selection for highly metastatic variants with gene expression profiling, Massagué and co-workers implicated miR-126, miR-206 and miR-335 as the first metastasis-suppressing miRNAs.[23] When ectopically expressed in breast cancer xenograft assays, these three miRNAs exerted unique effects on distinct aspects of the metastatic process: whereas miR-126 acted as a general inhibitor of tumor cell proliferation (at both the primary site and in distant organs), miR-206 and miR-335 instead inhibited cell motility and invasiveness.[23] Significantly, inhibition of miR-335 also augmented metastasis in vivo.[23] Additionally, in a study comparing metastasis-specific methylation of the promoters of human miRNA genes, the Esteller laboratory identified miR-34b/c and miR-148a as miRNAs whose overexpression was sufficient to impair the metastasis of human head and neck carcinoma xenografts by virtue of their ability to impair cell motility and invasiveness.[24]

While gain-of-function approaches (and loss-of-function studies, in the case of miR-335) in xenograft models have demonstrated that each of the aforementioned miRNAs was capable of altering metastatic capacity, it has remained unclear whether these in vivo effects could be attributed specifically to influences on one or more steps of the invasion-metastasis cascade; resolution of this question has been obscured by the potentially confounding reported influences of these miRNAs on primary tumor development, cell proliferation and/or apoptosis.[19,23-28] Hence, the extent to which miRNAs were capable of specifically regulating metastasis has remained unresolved.

Clarification of this issue was recently provided by our own studies regarding the role of miR-31 in altering the metastatic propensity of human breast cancer cells.[29] By deploying complementary gain-of-function and loss-of-function strategies, we demonstrated that miR-31 acts pleiotropically to suppress metastasis; these effects were achieved in the absence of confounding influences of this miRNA on primary tumor development.[29] Moreover, we documented that miR-31 intervened during multiple steps of the invasion-metastasis cascade in vivo, including effects on local invasion, one or more early post-intravasation events, and metastatic colonization.[29] Consequently, it is now clear that certain miRNAs are capable of specifically modulating the metastatic potential of human tumor cells grown as xenografts.

Table 1 summarizes our current knowledge regarding the identities of metastasis-relevant miRNAs, as well as their validated target genes, proposed broader mechanisms of action, and known correlations between their expression levels and clinical progression in human cancer patients. Not surprisingly, among the functionally relevant downstream effectors of these miRNAs are numerous previously characterized regulators of tumor cell invasion and metastasis, such as RhoA and tenascin C.[23,29] This suggests that miRNAs are likely to function as crucial, yet previously unappreciated, nodes within already-identified metastasis signaling circuitry.

Importantly, because an individual miRNA is capable of simultaneously regulating the expression of dozens of targets (and thus numerous signaling networks in parallel), deregulation of a single miRNA can affect the completion of multiple steps of the invasion-metastasis cascade. This, in turn, carries significant implications for our understanding of the pathogenesis of high-grade malignancies: the functional pleiotropy of individual miRNAs provides one explanation for how tumor cells can accumulate the requisite genetic and epigenetic aberrations needed to override the multiple safeguards that normally operate to prevent metastasis over the course of a typical human lifespan.

Integrating MicroRNAs into Emerging Paradigms of Metastatic Progression

Perhaps the most exciting consequence of these emerging roles for miRNAs involves the mechanistic insights that these regulators may provide into several recently described phenomena thought to play critical roles in one or more aspects of metastatic progression. Here, we highlight several such points of apparent convergence and speculate regarding potentially fruitful avenues of future research.

MicroRNAs and tumor-initiating cells. On the basis of research carried out over the last decade, some have proposed that only a sub-population of the neoplastic cells present in a tumor, the so-called "tumor-initiating cells" (TICs), possess the self-renewal capacity required to seed new tumors.[30] Indeed, xenograft serial transplantation studies involving several human tumor types lend support to this model, though the applicability of these findings to all types of human malignancies remains actively debated.[30] Of particular relevance to metastasis, the TIC hypothesis asserts that one or more TICs must disseminate from a primary tumor during the course of disease progression in order

Table 1. MicroRNAs implicated in the regulation of tumor metastasis

MicroRNA	Influence on metastasis	Experimentally validated downstream effectors(s)[1]	Step(s) of the invasion-metastasis cascade affected	Correlations with metastatic progression in human cancer patients	Ref.
miR-10b	Pro-metastatic	HoxD10	Cell motility and invasion	Upregulated in primary breast tumors that metastasized.[19] No association with metastasis in primary breast tumors.[62]	19, 58
miR-21	Pro-metastatic	Maspin, PDCD4, TPM1	Cell motility and invasion	High expression correlates with advanced stage, incidence of metastasis and/or poor outcome in breast and pancreatic tumors.	11, 21, 22
miR-31	Anti-metastatic	Fzd3, ITGA5, M-RIP, MMP16, RDX, RhoA	Cell motility and invasion; one or more early post-intravasation events; metastatic colonization	Downregulated in primary breast tumors that metastasized. Reduced expression in distant metastases, relative to patient-matched primary tumors.	29
miR-34b/c	Anti-metastatic	c-myc, CDK6, E2F3	Cell motility and invasion	Hypermethylated in primary breast, lung and colon tumors that formed metastases.	24
miR-126	Anti-metastatic	Unknown	General inhibitor of cell proliferation	Inversely associated with metastatic relapse in primary breast tumors.	23
miR-148a	Anti-metastatic	TGIF2	Cell motility and invasion	Hypermethylated in primary breast, lung and colon tumors that formed metastases.	24
miR-206	Anti-metastatic	Unknown	Cell motility and invasion	Downregulated in primary breast tumors that metastasized.	23
miR-335	Anti-metastatic	SOX4, TNC	Cell motility and invasion; tumor-initiating cell capacity?	Inversely associated with metastatic relapse in primary breast tumors.	23
miR-373/520c	Pro-metastatic	CD44, LATS2	Cell motility and invasion	Increased expression in metastases, relative to patient-matched primary breast tumors.	20, 25

[1]Experimentally validated by one or more standard in vitro assays for miRNA target validation (luciferase reporter assay, western blot and/or RT-PCR). CDK6, cyclin dependent kinase 6; ITGA5, integrin α5; LATS2, large tumor suppressor homolog 2; M-RIP, myosin phosphatase-Rho interacting protein; MMP16, matrix metallopeptidase 16; PDCD4, programmed cell death 4; RDX, radixin; TGIF2, TGFβ-induced factor homeobox 2; TNC, tenascin C; TPM1, tropomyosin 1.

for a macroscopic metastasis to develop; accordingly, if a non-TIC disseminates to a secondary locus, its limited self-renewal capacity precludes it from spawning a clinically significant, macroscopic metastasis.[31]

To date, only a limited number of functional regulators of the TIC-state have been identified; one such modulator includes the let-7 miRNA seed family.[32] In human breast cancer cells, ectopic expression of let-7 reduced self-renewal capacity and promoted differentiation; conversely, transient inhibition of let-7 promoted self-renewal.[32] Provocatively, let-7-expressing cells were also impaired in terms of their ability to metastasize.[32] However, let-7 is also known to function in anti-mitogenic and pro-apoptotic capacities via repression of downstream effectors that include the Ras, c-myc and HMGA2 oncogenes;[10] this confounds interpretation of the reported influences of let-7 on TIC capacity and metastatic dissemination.

Interestingly, the anti-metastatic miR-335 directly represses SOX4—a transcription factor important for the maintenance of a progenitor cell-like state—in human breast cancer cells.[23] Somewhat more puzzling is the finding that the metastasis-promoting miR-373/520c downregulates the levels of CD44,[20] a cell-surface antigen known to be highly expressed in TIC-enriched sub-fractions of human breast cancer cells, in a mammary carcinoma model.[33]

Future studies will be needed to identify additional miRNAs that affect metastatic propensity by either supporting or antagonizing the TIC-state. Moreover, it will be important to pinpoint more precisely the observed effects on metastasis that arise specifically from perturbation of TIC function, distinguishing these from changes in metastatic behavior that derive from confounding influences on general cell proliferation, resistance to apoptosis, or the completion of various early steps of the invasion-metastasis cascade such as local invasion.

MicroRNAs and the epithelial-mesenchymal transition. The epithelial-mesenchymal transition (EMT) is an evolutionarily conserved developmental program that converts otherwise-immotile epithelial cells into mesenchymal cells that possess a high migratory capacity.[34,35] The EMT circuitry can also be co-opted opportunistically by carcinoma cells, enabling them to complete multiple steps of the invasion-metastasis cascade.[34,35] miR-200

has been identified as a miRNA seed family whose expression is both necessary and sufficient to oppose induction of an EMT via downregulation of the EMT-promoting transcription factors ZEB1 and ZEB2.[36-40] Members of the miR-200 family suppress motility and invasiveness in several cancer cell types in vitro;[36-40] however, the impact of modulating levels of miR-200 on in vivo metastasis has yet to be reported.

Similarly, miR-205 protects renal cells from TGFβ-mediated EMT,[36] although the functional consequences of either gain or loss of miR-205 expression on in vitro surrogates of metastatic capacity and in vivo metastasis remain to be clarified. Additionally, the pro-metastatic miR-10b was shown to be a direct transcriptional target of the EMT-inducing transcription factor Twist1; however, ectopic expression of miR-10b, on its own, failed to induce an EMT in human breast cancer cells.[19] Together, the preceding observations suggest that certain miRNAs are deeply embedded within signaling networks known to be important for induction of an EMT, some of which appear to be relevant to tumor metastasis.

While the EMT machinery can undoubtedly facilitate metastatic dissemination, certain miRNAs appear to affect metastasis without functioning as components of the EMT program. miR-126, miR-206 and miR-335 exert their anti-metastatic abilities without reversing the EMT status of human breast cancer cells.[23] Similarly, our own data indicate that miR-31 opposes metastasis without altering the expression of established markers of the EMT and that endogenous levels of miR-31 are not controlled by the EMT modulators Twist1, Snail1, ZEB1 and E-cadherin (Valastyan S and Weinberg RA, unpublished observations).

Several pressing questions regarding the emerging convergence between the induction of an EMT, the expression levels of specific miRNAs, and the regulation of metastasis remain to be addressed. For example, demonstration that miR-200 affects the metastatic capabilities of in vivo tumor xenografts is imperative. Second, it will be critical to demonstrate that any observed effects of miR-200 family members on in vivo metastasis are specifically attributable to the capacity of these miRNAs to suppress EMT regulatory circuitry, rather than affecting additional cell phenotypes that are mediated by mechanistically unconnected downstream effectors. Third, recent work from our laboratory has described an unexpected connection between the induction of an EMT and the acquisition of TIC-like attributes in human breast cancer cells.[41] Consequently, it is tempting to speculate that miRNAs known to affect TIC functions (such as let-7) may also play integral roles in modulating the EMT program; similarly, miRNAs that impinge upon EMT circuitry may also regulate the TIC-state. In accord with this supposition, miR-200c—a known antagonist of the EMT program[36-40]—has recently been proposed to impair the TIC capacity of human breast cancer cells.[42] Finally, while miR-10b, miR-31, miR-126, miR-206 and miR-335 do not appear to alter the levels of well-established EMT markers,[19,23] it will be important to clarify whether these potent regulators of metastasis actually operate independently of the EMT circuitry or instead contribute to certain aspects of transient and/or context-dependent EMT programs. Resolution of this last issue may provide insight as to whether induction of an EMT is a universal, integral component of all metastasis-promoting mechanisms or instead is only one of several alternative cell-biological programs deployed by carcinoma cells to acquire the ability to disseminate and colonize distant tissue sites.

MicroRNAs and organ-specific metastatic colonization. More than 100 years ago, Stephen Paget articulated his "seed and soil" hypothesis of metastatic outgrowth based upon clinical observations detailing preferential metastasis of a given type of cancer to one or more particular distant organ sites.[43] This viewpoint posits that, while tumor cells are widely disseminated during the course of malignant progression, detectable metastases only develop at those sites ("soils") where the tumor cells ("seeds") are suitably adapted for survival and proliferation.[43] While the anatomical layout of the vasculature is likely responsible for certain stereotypical patterns of metastasis,[3,44] evidence from a number of laboratories—notably the work of Fidler and colleagues—has documented that specific organ microenvironments are indeed intrinsically more or less hospitable to certain types of disseminated tumor cells.[1,2] In humans, this was most vividly demonstrated by observing ovarian cancer patients provided palliative remediation via the insertion of peritoneovenous shunts.[45] In addition to relieving pain, this treatment—which evacuates ascites fluid into the venous circulation—liberated millions of cancer cells into the systemic circulation; nevertheless, these patients largely failed to develop detectable metastases even several years after the installation of the shunts.[45]

More recently, the Massagué laboratory has identified a number of protein-encoding genes whose expression facilitates breast cancer metastasis specifically to either bone,[46] lungs[47] or brain.[48] Ostensibly, these factors favor outgrowth in an organ-specific manner due to their ability to allow disseminated tumor cells to overcome certain obstacles to metastasis formation imposed by the tissue microenvironment of that particular organ.[46-48]

Interestingly, whereas many metastasis-relevant protein-encoding genes only impinge upon outgrowth at a single distant site (e.g., a gene promotes lung metastasis but has no effect on bone metastasis),[1,46-48] several miRNAs have been reported to contribute to more widespread, multi-organ metastasis. For example, miR-10b overexpression increased the incidence of both pulmonary and peritoneal metastases in breast cancer xenograft models.[19] Similarly, miR-126, miR-206 and miR-335 suppressed breast cancer metastasis to both bone and lungs.[23]

It is reasonable to speculate that these individual miRNAs simultaneously regulate multiple distinct downstream effectors that, in turn, facilitate the colonization of several different organs. Alternatively, such observations may merely indicate that these miRNAs impinge upon metastasis at an early step of the invasion-metastasis cascade—such as local invasion or survival in the vasculature—prior to the juncture at which organ-selectivity manifests itself. Further experimentation is required to distinguish between these possibilities. Moreover, the extent to which this is a property of additional miRNAs remains unresolved, as the ability or inability of other metastasis-relevant miRNAs to affect multi-organ metastasis has not yet been reported.

MicroRNAs and the functional plasticity afforded by distinct tumor cell invasion mechanisms. Tumor cells exploit

multiple alternative molecular mechanisms to acquire the capacity for cellular migration and tissue invasion,[49] both of which represent prerequisites for metastasis formation. More specifically, tumor cells are capable of acquiring invasiveness via executing both protease- and integrin-dependent "mesenchymal" motility programs, as well as protease- and integrin-independent "amoeboid" motility programs.[49] Tumor cells can shift from one form of invasion to the other in response to environmental challenges (for example, treatment with broad spectrum protease inhibitors); consequently, some have proposed that abrogation of tumor cell invasion can only be achieved upon concomitant inhibition of both the mesenchymal and amoeboid motility circuitries.[49]

As previously discussed, several miRNAs have been documented to exert strong influences on tumor cell invasion and migration. These potent effects are ostensibly attributable to the capacity of certain miRNAs to pleiotropically regulate multiple downstream targets that include core components of both mesenchymal and amoeboid invasion signaling networks. Empirical evidence for this supposition is provided by the apparent mechanism of action utilized by miR-31 to achieve attenuation of cell motility and invasiveness:[29] this miRNA downregulates key effectors of both the mesenchymal program (including integrin α5 and MMP16[49]) and the amoeboid program (including RhoA[49]). Hence, miR-31's potent inhibitory influences on motility and invasion may derive from its pleiotropic capacity to simultaneous impair the mesenchymal and amoeboid invasion programs.

We speculate that analogous modes of action will eventually be described for other motility- and invasion-relevant miRNAs. Alternatively, it is possible that, instead of simultaneously repressing the mesenchymal and amoeboid invasion pathways, certain miRNAs may abrogate one of these two pathways while simultaneously impairing the ability of these cells to transition from one motility program to the other.

Metastasis-Relevant MicroRNAs as Prognostic and Therapeutic Tools for Human Cancers

Of fundamental importance is the question of whether insights gleaned from studying the roles of miRNAs in tumor metastasis will prove useful to the diagnosis and treatment of human malignancies. Details regarding known correlations between the expression of specific miRNAs and metastatic progression in human cancer patients are summarized in **Table 1**.

One unexpected finding in this realm stems from our investigations of miR-31 expression in primary human breast tumors, where we discovered that miR-31's inverse association with metastasis persisted independent of both tumor grade and molecular subtype.[29] Such grade- and subtype-independence was surprising, as existing prognostic markers for breast cancer largely correlate with tumor grade and/or molecular subtype; moreover, these previously identified biomarkers fail to stratify patients within the more aggressive basal-like or $HER2^+$ subtypes who are at especially high risk of clinical progression.[50] This raises the interesting possibility that prognostic signatures derived from miRNA expression patterns may contain additional information that is not present in mRNA expression signatures.

Levels of both miR-373[20] and miR-31[29] differ between patient-matched primary breast tumors and distant metastases disseminated from those primary tumors. This is noteworthy, since evidence of gene expression changes occurring during the course of disease progression within an individual patient has only rarely been observed for protein-encoding genes important for metastasis.[51] If similar observations are made with other metastasis-relevant miRNAs, this may reconcile the observation that only a relatively modest number of protein-encoding genes exhibit changes in expression level between paired primary tumors and metastases with the widely held belief that additional genetic and/or epigenetic lesions are required to confer metastatic proficiency upon tumorigenic but still non-metastatic cells.[1-3,51]

We believe that it is likely that certain miRNAs will eventually be identified as highly useful prognostic biomarkers for predicting susceptibility to metastasis in a variety of human neoplasias. What is less clear at present, however, is the utility of miRNAs in the development of novel therapies. For example, will therapeutic benefit derive from manipulation of miRNA levels in malignant human tumors, achieved either by restored expression of metastasis-suppressing miRNAs or inhibition of pro-metastatic miRNAs?

To be sure, miRNAs represent attractive therapeutic targets due to their capacity to pleiotropically regulate entire cohorts of genes that, in turn, impact multiple neoplastic phenotypes. However, in addition to difficulties associated with inadvertent toxicity to normal cells, major impediments have been encountered because of the inherent metabolic instability of RNA molecules and problems with effective delivery of the therapeutic agent to the tumor cells.[52] Despite these obstacles, it is encouraging that "antagomirs"[23] (lipid-modified antisense inhibitors of miRNA function[53]) and adeno-associated virus-mediated delivery of miRNA-encoding sequences[54] have demonstrated clear therapeutic benefits in murine cancer models. Ongoing efforts to improve these methodologies may one day result in the derivation of truly efficacious anti-cancer therapies.

Future Outlook

Remarkable progress has been made regarding the roles of miRNAs in tumor metastasis over a relatively short interval. As this field continues to develop, it is increasingly important to demonstrate the relevance of individual miRNAs to tumor cell dissemination in vivo, as available in vitro surrogates for metastatic attributes fail to recapitulate the full complexity of in vivo metastasis. Ideally, genetic murine models will soon become available, in which animals deficient for metastasis-relevant miRNAs will be crossed into tumor-prone backgrounds and metastasis phenotypes then assessed.

It is now necessary to obtain more detailed insights into the precise mechanisms-of-action of metastasis-associated miRNAs; more specifically, it is of significant interest to identify through which particular downstream effectors these miRNAs act to exert their effects on metastasis. Such mechanistic understanding is best attained through re-expression of cDNA constructs

encoding certain downstream targets that have been rendered miRNA-insensitive via deletion of 3'UTR sequences in cells co-expressing the miRNA of interest. Once again, these investigations can only provide unequivocal insight when they include an in vivo assay for metastasis development.

Importantly, miRNAs are only the first class of an extensive family of diverse non-coding RNAs (ncRNAs) whose levels are likely to be altered in a functionally important manner during pathological states such as malignancy.[9] Indeed, recent work from the Croce laboratory has identified novel species of ncRNAs whose expression are altered in certain human leukemias and carcinomas.[55] We anticipate that the biology of these ncRNAs—as well as additional classes of ncRNAs—will further our understanding of the pathogenesis of high-grade malignancies.

The activities of specific miRNAs may also help to explain the mechanistic basis underlying still-emerging paradigms within the larger field of metastasis research, including the early dissemination of pre-neoplastic cells.[56,57] Assessed collectively, interrogation and modulation of miRNA levels hold great promise in terms of facilitating improved diagnosis and treatment of human tumors, as the pleiotropic activities of miRNAs place them as central nodes within integral metastasis-regulating signaling circuitry.

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Assaying microRNA loss-of-function phenotypes in mammalian cells

Emerging tools and their potential therapeutic utility

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MicroRNAs are small, non-coding RNAs that are increasingly appreciated to play critical roles in the modulation of gene expression. In mammalian cells, our knowledge regarding the full impact of microRNAs on cellular behavior remains fragmentary. This has been due, in significant part, to the limited availability of experimental tools for studying microRNA loss-of-function phenotypes. Recently, several strategies for achieving this goal have been developed. Here, we discuss these methodologies for inhibiting specific microRNAs in mammalian cells both in vitro and in vivo, compare and contrast the strengths and weaknesses of these approaches, and speculate regarding the future impact of these antagonists on the treatment of human diseases such as cancer. These emerging techniques enable the attenuation of microRNA function in a manner that is quite sequence-specific, relatively long-lasting and increasingly cost-effective. As such, some of these advances hold great promise in terms of their eventual utility as therapeutic agents.

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MicroRNAs (miRNAs) are an evolutionarily conserved family of pleiotropically acting regulatory RNAs that inhibit gene expression post-transcriptionally.[1] Although originally identified through forward genetic screens in the nematode *C. elegans*,[2,3] miRNAs have since been found to be expressed in a wide variety of eukaryotes.[4] Indeed, more than 650 distinct miRNAs have now been identified in human cells.[4] Because each individual miRNA can modulate the expression of numerous distinct mRNA targets, it has been proposed that greater than one-third of the mRNA species encoded in the human genome are likely to be regulated by miRNAs.[4] Several excellent review articles have recently been published that summarize our current understanding of miRNA biogenesis, miRNA targeting specificity, and the mechanisms by which miRNAs silence gene expression post-transcriptionally;[4,5] accordingly, we will not discuss these topics here.

Research conducted over the past decade has implicated specific miRNAs as critical regulators of numerous normal cell-physiologic processes in a variety of organisms, including humans.[1] Moreover, aberrant miRNA activity has been documented to contribute to the pathogenesis of many disease states.[1,6,7] While remarkable progress has been made over a relatively short period of time, our knowledge regarding the precise functions of miRNAs has suffered from a dearth of techniques that can be utilized to inhibit specific miRNAs in mammalian cells.[8,9]

The murine germ-line knockout procedure[10] and RNA-interference technology[11] revolutionized investigations of the functions of protein-encoding genes by enabling study of their loss-of-function phenotypes in mammalian cells. Excitingly, several recent reports have now described novel means by which to selectively attenuate the function of individual miRNAs. We envision that refinement of these miRNA loss-of-function approaches will greatly augment our understanding of the biological activities of miRNAs.

Table 1. Available methodologies for assaying microRNA loss-of-function phenotypes in mammalian cells

Category	Technique	Specificity of repression	Duration of inhibition	Efficacy in vitro and in vivo	Associated monetary costs	Ref.
Antisense oligonucleotides						
	2'-O-methyl-ribonucleotides	Individual miRNA	Several days	Effective only in vitro	Significant (repeated administration required)	12, 13
	Antagomirs	Individual miRNA	Several weeks	Effective both in vitro and in vivo	Significant (repeated administration required)	14
	LNA oligonucleotides	Individual miRNA	Several weeks	Effective both in vitro and in vivo	Significant (repeated administration required)	15
microRNA sponges						
	Transfected sponges	Entire miRNA seed family	Several days	Effective only in vitro	Relatively modest	16
	Virally expressed sponges	Entire miRNA seed family	~Permanent	Effective both in vitro and in vivo	Relatively modest	17–22
Genetic murine models						
	Traditional knockout mice	Individual miRNA; Several adjacent miRNAs	Permanent	By definition, only implemented in vivo	Significant	23–28
	Sponge-expressing transgenic mice	Entire miRNA seed family	~Permanent	By definition, only implemented in vivo	Significant	None yet reported

LNA, locked-nucleic-acid; miRNA, microRNA.

In the discussions that follow, which are summarized in **Table 1**, we highlight a number of emerging strategies for inhibiting individual miRNAs. For each of these procedures, we explore four critical parameters: (i) the extent to which the technique specifically silences a single miRNA, (ii) the duration of the achieved inhibitory effect, (iii) whether the technology is efficacious both in vitro and in vivo, and (iv) the financial costs associated with implementation. When assessed collectively, these four parameters afford insight concerning not only the robustness of the technique, but also its putative future application as a means of therapeutic intervention.

The first described strategies to achieve functional silencing of miRNAs involved the direct in vitro introduction of short oligonucleotides that shared extensive sequence complementarity with the miRNA of interest; these antisense molecules therefore acted as stoichiometric competitive inhibitors of miRNA activity by directly binding the targeted miRNA.[8,12,13] Initially, 2'-O-methyl-ribonucleotides were employed as the preferred inhibitory molecules; however, the duration of inhibition achieved upon transfection of these antagonists in vitro was found to be relatively modest—typically on the order of only several days.[8,13] In order to remedy this shortcoming and thus attain more long-lasting attenuation of miRNA function, chemical modifications were introduced into the nucleotide backbone of the antisense inhibitors.[8,9,14,15] For example, Stoffel and colleagues developed "antagomirs"—cholesterol-conjugated single-stranded RNA molecules capable of attenuating the activity of a targeted miRNA for several weeks both in vitro and in vivo.[14] Similarly, locked-nucleic-acid (LNA)-modified oligonucleotides have been utilized to effectively silence the actions of a specific miRNA in vitro and in vivo for multiple weeks.[15]

The antisense inhibitors cited above display exquisite sequence-specificity in terms of their targeting, with only a small number of single-nucleotide substitutions rendering the antagonists biologically inert.[8,14] Moreover, optimization of the structure of the antisense molecule at sites outside of the segment containing the sequence complementary to the miRNA target resulted in inhibition that was relatively long-lasting;[14,15] importantly, this facilitated effective miRNA silencing not only in vitro, but also in vivo. Nonetheless, adequate inhibition of miRNA function often requires the repeated administration of relatively large doses of the inhibitory molecule;[8,14,15] this results, in turn, in a substantial expense, given the significant costs associated with purchasing these reagents. To summarize, the various antisense inhibitory molecules described above provide a useful means for antagonizing specific miRNAs in vitro; additionally, certain chemically modified oligonucleotide inhibitors are also efficacious in vivo.

An alternative strategy to achieve functional silencing of miRNAs involves the plasmid-based expression of tandemly arrayed nucleotide sequence motifs complementary to the miRNA of interest. These "miRNA sponges" (also known as "miRNA decoys") act as competitive inhibitors of miRNA function by binding

the targeted miRNA and thereby diverting it from its normal cognate mRNA targets.[9,16-22] At least three parameters are of critical importance to the efficacy of these constructs: (i) the strength of the promoter utilized to drive expression of the sponge transgene, (ii) the number of tandem binding sites complementary to the miRNA of interest that are included in the sponge vector construct and thus in each sponge RNA molecule, and (iii) the extent of sequence complementarity shared between the sponge motifs and the targeted miRNA.[9,16] Nucleotide mismatches are often introduced at several positions along the length of the sponge-expressed complementary sequences to block Argonaute2-catalyzed cleavage of the miRNA-bound RNA duplex; this, in turn, augments the half-life of the inhibitory complex by preventing its turnover.[16] Taken together, these considerations have led some to propose that effective miRNA silencing requires very strong promoter elements driving the expression of a sponge construct containing at least six imperfectly complementary binding motifs.[9,16]

An initial miRNA sponge design from the Sharp laboratory relied upon transient transfection of sponge-expressing sequences; these constructs were capable of inhibiting miRNA function over a period of several days in vitro.[16] Subsequently, adenoviral,[17] lentiviral[18-21] and retroviral[22] vectors have been employed to drive expression of miRNA sponge constructs, resulting in robust inhibition of the desired miRNA both in vitro and in vivo. Owing to the ability of these viral vector-transduced sponge sequences to stably integrate into the host cell genome, the inhibitory effects of these viral vector miRNA sponges persist for significantly longer periods of time than transfected antisense oligonucleotide inhibitors, antagomirs, LNA oligonucleotides and transiently transfected miRNA sponges.[9,17-22] Ostensibly, these viral vector-expressed sponges should silence the targeted miRNA in the initially infected cell and its descendents and continue to do so for an indefinite interval. We found, for example, that a retrovirus vector-transduced miRNA sponge targeting miR-31 in human breast cancer cells retained efficacy over the course of a four-month xenograft assay in vivo.[22]

Of note, whereas antisense inhibitors are designed to uniquely target only a single miRNA, miRNA sponges exhibit slightly broader antagonistic functions by simultaneously inhibiting all members of the same miRNA seed family (i.e., those miRNAs sharing identical sequence at nucleotide positions 2–8 from the 5' end of the mature miRNA—a region that is absolutely critical for determining targeting specificity[4]).[9,16] This aspect of miRNA sponge biology provides a means by which to concurrently inhibit all members of large families of closely related miRNAs whose number of paralogs renders them resistant to alternative silencing strategies, all of which impact only a single specific miRNA—for example, the let-7 family, which is comprised of 11 members in human cells.[16] As described above, modification of the miRNA sponge technology to allow for viral vector-transduced sponge expression affords long-term repression of the targeted miRNA,[9,17-22] thus making miRNA sponges a useful tool for the repression of miRNA function both in vitro and in vivo. Finally, because miRNA sponges are expressed from DNA plasmids, rather than existing as short-lived directly transfected oligonucleotides, the financial costs imposed by sponge-mediated inhibition of miRNA function are substantially less than those associated with transfected antisense inhibitors. Consequently, when assessed collectively, recently reported evidence indicates that miRNA sponges are cost-effective tools for the stable suppression of miRNA function both in vitro and in vivo.

A third technique by which to abolish the function of a desired miRNA involves the creation of in vivo murine models deficient for the miRNA of interest, this being achieved via classical gene knockout techniques.[10] In contrast to the strategies described above, genetic knockouts are unique in terms of their ability to entirely eliminate the function of a targeted miRNA, rather than incompletely reducing its levels.[8-10] This approach is compromised, however, by the fact that (i) some miRNAs are encoded by several distinct loci within the genome, (ii) multiple miRNAs often exist that share the same seed sequences (and thus redundantly target the same mRNA effectors), and (iii) many miRNA genes are encoded within introns of protein-encoding genes such that deletion of the miRNA might introduce a confounding variable via compromising the functionality of the surrounding protein-encoding gene;[1,4,5] hence, certain miRNAs may not be amenable to traditional gene knockout analysis. Instead, in vivo murine models for attenuated function of such miRNAs might require the creation of transgenic animals expressing miRNA sponge constructs that antagonize an entire miRNA seed family under investigation.

To date, only a small number of murine miRNA-encoding genes have been manipulated by genetic knockout technologies.[23-28] From these reports, it is apparent that mammalian miRNAs are likely to play essential and largely non-redundant roles in normal cell or organismal physiology;[23-28] this contrasts with the modest phenotypes elicited by the genetic ablation of individual miRNA genes in *C. elegans*, where the presence of numerous miRNA genes with similar seed sequences apparently masks the effects deriving from single-gene knockouts.[29] In the case of mammals, one such example of the critical role of miRNAs in normal development is provided by the reported phenotype of the miR-208 knockout mouse.[24] This work revealed that miR-208 is required for stress-dependent cardiac growth and the induction of normal cardiomyocyte gene expression programs in vivo.[24] These findings—as well as the published phenotypes of the miR-1-2,[23] miR-155,[25,26] miR-223,[27] and miR-17~92 cluster[28] knockout mice—are certainly provocative and provide a strong impetus for the creation of additional miRNA-deficient knockout mice.

By definition, gene targeting as a strategy to eliminate miRNA function is highly specific for a single genomic locus.[10] However, as elegantly demonstrated by the Jacks laboratory in their studies concerning the role of the miR-17~92 cluster, a single targeting procedure can be utilized to concomitantly delete several miRNA genes positioned within close proximity to one another in tandem along a chromosome.[28] Genetic knockouts provide permanent eradication of the function of the targeted miRNA, although conditional targeting strategies employing the

Cre/loxP or FLP/FRT systems can enable temporal control over the genetic ablation. Additionally, the utilization of tissue-specific promoters to drive Cre and FLP expression can provide an additional layer of spatial control to the induced genetic manipulation.[10] In light of the fact that germ-line deletion of certain miRNAs is likely to yield an embryonic lethal phenotype due to the presumed vital role of specific miRNAs in essential developmental processes, these opportunities for temporal and spatial control may prove critical for many future studies investigating the roles of miRNAs in adult physiology in vivo. Finally, one serious disadvantage of assaying miRNA loss-of-function phenotypes by genetic knockout techniques involves the significant financial costs associated with the implementation of these technologies, as well as the long period of time required to generate the genetically deficient animals;[10] we speculate that certain aspects of these impediments could be ameliorated via the creation of transgenic animals expressing miRNA sponges instead of true genetic knockout mice. Together, available evidence suggests that eliminating the function of specific miRNAs via gene knockout technologies will provide valuable insight regarding their biological niches in vivo.

Table 1 summarizes the currently available mammalian miRNA loss-of-function techniques delineated above, comparing and contrasting (i) their specificity, (ii) the duration of silencing elicited, (iii) their effectiveness both in vitro and in vivo, and (iv) the financial costs associated with each approach. Given the particular pros and cons associated with each of these strategies, different techniques will likely prove to be more effective for addressing different biological questions.

In addition to the basic scientific insight deriving from the above-described miRNA loss-of-function technologies, it is plausible that inhibiting specific miRNAs using these techniques may eventually prove useful in the treatment of various human diseases. For example, aberrant expression of numerous miRNAs has been observed in a variety of human tumors, and functional studies have demonstrated critical roles for many specific miRNAs in myriad aspects of tumorigenesis.[6,7,22] Indeed, it has been posited that miRNAs are likely to function as critical nodes within already-identified neoplastic signaling circuitry, and that their profound impact on malignant progression arises from the capacity of individual miRNAs to pleiotropically regulate dozens of distinct effector mRNAs.[7,22] Consequently, certain cancer treatments developed in the future may depend upon the inhibition of pro-tumorigenic miRNAs.

We envision that several of the miRNA loss-of-function tools described herein may possess clinical utility. Indeed, antagomirs and LNA oligonucleotides have already been shown to be relatively stable and long-lived within the circulation and organs of mammals, including non-human primates.[14,15] Additionally, optimized miRNA sponge constructs are also likely to efficiently antagonize individual miRNAs over extended intervals of time in vivo. However, significant obstacles remain to be surmounted before miRNA antagonists begin to yield clinical benefits. At present, major difficulties arise due to inadvertent toxicity to normal cells, the inherent metabolic instability of RNA molecules, and the effective delivery of the therapeutic agent to targeted tissues.[8] Ongoing efforts to address these problems may one day result in the derivation of truly effective therapies.

As might be expected, not all pathological states resulting from altered miRNA levels arise due to the increased expression of particular miRNAs; it has also been documented that that the loss or down-regulation of certain miRNAs can elicit disease phenotypes.[6,7,22] From a clinical standpoint, it is therefore encouraging that adeno-associated virus-mediated delivery of miRNA-encoding sequences was recently reported to elicit clearly discernable therapeutic benefits in a murine cancer model in vivo.[30]

Overall, despite substantial progress over the course of the past decade, a number of fundamentally important issues surrounding mammalian miRNA biology have yet to be resolved. Many of these topics have previously remained uninvestigated due to the absence of robust techniques for assaying miRNA loss-of-function phenotypes in mammalian cells.[8] However, the recent development of several experimental strategies that enable inhibition of miRNA function in a highly specific, relatively long-lasting, and cost-effective manner both in vitro and in vivo are likely to remove major obstacles. For this reason, we anticipate that deployment of these tools will greatly enhance our knowledge of miRNA biology in the near future and, in the longer term, afford truly efficacious therapeutic opportunities.

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Concomitant suppression of three target genes can explain the impact of a microRNA on metastasis

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It remains unclear whether a microRNA (miRNA) affects a given phenotype via concomitant down-regulation of its entire repertoire of targets or instead by suppression of only a modest subset of effectors. We demonstrate that inhibition of breast cancer metastasis by miR-31—a miRNA predicted to modulate >200 mRNAs—can be entirely explained by miR-31's pleiotropic regulation of three targets. Thus, concurrent re-expression of integrin-α5, radixin, and RhoA abrogates miR-31-imposed metastasis suppression. These effectors influence distinct steps of the metastatic process. Our findings have implications concerning the importance of pleiotropy for the biological actions of miRNAs and provide mechanistic insights into metastasis.

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MicroRNAs (miRNAs) are an evolutionarily conserved family of regulatory RNAs that inhibit their mRNA targets post-transcriptionally, leading to modulation of diverse biological processes, including the development and progression of cancer (Ambros 2004; Bartel 2009; Ventura and Jacks 2009). An individual miRNA is capable of regulating dozens of distinct mRNAs (Baek et al. 2008; Selbach et al. 2008), and it is thought that pleiotropic suppression of multiple downstream effectors may underlie the phenotypic changes observed upon perturbing the levels of certain miRNAs (Rodriguez et al. 2007; Thai et al. 2007; van Rooij et al. 2007; Zhao et al. 2007; Johnnidis et al. 2008; Ventura et al. 2008). It remains unclear, however, whether these consequences depend on simultaneous deregulation of the entire repertoire of targets of a given miRNA or instead on the altered activity of only a small subset of effectors.

Metastases, which are responsible for 90% of human cancer deaths, arise via a complex series of events, collectively termed the invasion–metastasis cascade (Fidler 2003; Gupta and Massagué 2006). In order to metastasize, cells in a primary tumor must become motile, degrade surrounding extracellular matrix (local invasion), intravasate into the vasculature, retain viability during transit through the circulation, extravasate into the parenchyma of a distant tissue, survive in this foreign microenvironment to form micrometastases, and, finally, thrive in their new milieu and establish macroscopic secondary tumors (colonization) (Fidler 2003). Colonization is the rate-limiting step of the invasion–metastasis cascade, yet the molecular underpinnings of this process are poorly understood (Gupta and Massagué 2006).

We determined recently that expression of the miRNA miR-31 was both necessary and sufficient to inhibit the metastasis of human breast cancer xenografts, and that miR-31 levels correlated inversely with metastatic relapse in breast carcinoma patients (Valastyan et al. 2009). We attributed these effects to miR-31's ability to pleiotropically suppress a cohort of prometastatic targets; however, we did not identify a minimal set of downstream effectors whose concomitant re-expression is sufficient to fully override miR-31's influences on metastasis. For this reason, we sought to determine whether the impact of miR-31 on metastasis could be explained by its ability to pleiotropically modulate a defined subset of its >200 predicted targets.

Results and Discussion

We demonstrated previously that miR-31 regulates six mRNAs that encode proteins with roles in cell motility and tumor progression: frizzled3 (Fzd3), integrin-α5 (ITGA5), matrix metallopeptidase 16 (MMP16), myosin phosphatase-Rho-interacting protein (M-RIP), radixin (RDX), and RhoA (Valastyan et al. 2009). To begin to address whether miR-31-imposed inhibition of one or more of these effectors might be responsible for mediating miR-31's anti-metastatic influences, we stably suppressed these six mRNAs individually in otherwise metastatic MDA-MB-231 human breast cancer cells ("231 cells") using shRNAs. 231 cells are largely devoid of endogenous miR-31 and robustly express these six effectors; moreover, ectopic miR-31 impairs metastasis by these cells (Valastyan et al. 2009).

For each gene, we derived multiple cell lines that stably expressed a distinct shRNA targeting unique sequences in the encoded mRNA in order to minimize confounding influences from shRNA off-target effects (Supplemental Figs. 1A, 2A). At least one shRNA against each of the six effectors reduced its target's level by a factor comparable with that elicited by miR-31 expression (Valastyan et al. 2009). This allowed us to reasonably approximate the consequences of miR-31's actions on each individual downstream effector.

These shRNA-expressing 231 cells were subjected to in vitro assays that model traits important for metastasis. We observed that individual suppression of ITGA5, RDX, or RhoA reduced invasion, motility, and resistance to

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anoikis-mediated cell death in vitro; in contrast, the Fzd3, MMP16, or M-RIP shRNAs failed to substantially affect these behaviors (Supplemental Figs. 1B–D, 2B–D). For shRNAs that conferred measurable responses, the magnitude of these responses was directly correlated with the extent of knockdown achieved, suggesting that these effects arose as a specific consequence of reduced levels of the targeted protein. Inhibition of Fzd3, ITGA5, MMP16, M-RIP, RDX, or RhoA failed to affect in vitro proliferation (Supplemental Figs. 1E, 2E). Also, the responses evoked by the ITGA5, RDX, and RhoA shRNAs could not be ascribed to saturation of the miRNA biogenesis machinery, as mature levels of eight control miRNAs were unaffected in these cells (Supplemental Fig. 3).

We determined whether suppression of these six mRNAs altered metastatic capacity in vivo by intravenously injecting the shRNA-expressing 231 cells into mice. One month later, cells bearing shRNAs targeting ITGA5, RDX, or RhoA had generated 80%, 85%, and 55% fewer lung metastases than controls, respectively; however, down-regulation of Fzd3, MMP16, or M-RIP did not affect the number of metastases spawned (Supplemental Fig. 4). Thus, inhibition of ITGA5, RDX, or RhoA—but not Fzd3, MMP16, or M-RIP—affects in vitro surrogates of metastatic capacity as well as in vivo metastasis.

To extend these analyses, we stably re-expressed miRNA-insensitive versions of the mRNAs encoding Fzd3, ITGA5, MMP16, M-RIP, RDX, or RhoA individually in 231 cells that already expressed either miR-31 or control vector (Supplemental Fig. 5A). This allowed us to gauge the ability of each of these effectors—when re-expressed—to reverse miR-31's impact on in vivo metastasis. When introduced into the venous circulation of mice, miR-31-expressing cells formed 85% fewer lung metastases than controls 1 mo post-injection (Supplemental Fig. 5B), consistent with our prior findings (Valastyan et al. 2009). Individual re-expression of ITGA5, RDX, or RhoA restored the number of lung metastases in miR-31-expressing cells to 55%, 50%, and 65% of control levels, respectively; in contrast, Fzd3, MMP16, or M-RIP failed to increase lesion number (Supplemental Fig. 5B). Overexpression of ITGA5, RDX, or RhoA did not further enhance metastasis in control 231 cells (Supplemental Fig. 5B), suggesting that signaling from these pathways was already saturated in 231 cells, as has been established previously for RhoA-controlled networks (Pillé et al. 2005). Together, these findings implied that, although miR-31 is capable of suppressing numerous mRNA species, its ability to regulate only a subset of these effectors appears to be crucial for its capacity to impair metastasis.

In support of this notion, when stably re-expressed in 231 cells, Fzd3, MMP16, or M-RIP failed to reverse miR-31-imposed attenuation of invasion, motility, and anoikis resistance in vitro (Supplemental Fig. 6); in contrast, our prior work revealed that restored levels of ITGA5, RDX, or RhoA rescued, at least partially, miR-31-evoked defects in these phenotypes (Valastyan et al. 2009). Based on these in vitro and in vivo re-expression data, as well as the above-described in vitro and in vivo loss-of-function findings, we focused our subsequent analyses on the ability of inhibition of ITGA5, RDX, and RhoA to account for miR-31's anti-metastatic activities.

To this end, we investigated the consequences of suppressing ITGA5, RDX, or RhoA individually in an orthotopic injection assay. Accordingly, we implanted 231 cells expressing shRNAs targeting either ITGA5, RDX, or RhoA into the mammary fat pads of mice. Suppression of ITGA5 or RhoA did not affect primary tumor growth; conversely, inhibition of RDX reduced the size of resulting mammary tumors (Fig. 1A). After normalizing for differences in primary tumor growth, cells expressing shRNAs against ITGA5, RDX, or RhoA formed 85%, 70%, and 50% fewer lung metastases than controls 2.5 mo after injection, respectively (Fig. 1B). Thus, inhibition of ITGA5, RDX, or RhoA each impedes metastasis; however, this assay did not reveal the particular step(s) of the invasion–metastasis cascade that were impaired due to suppression of ITGA5, RDX, or RhoA.

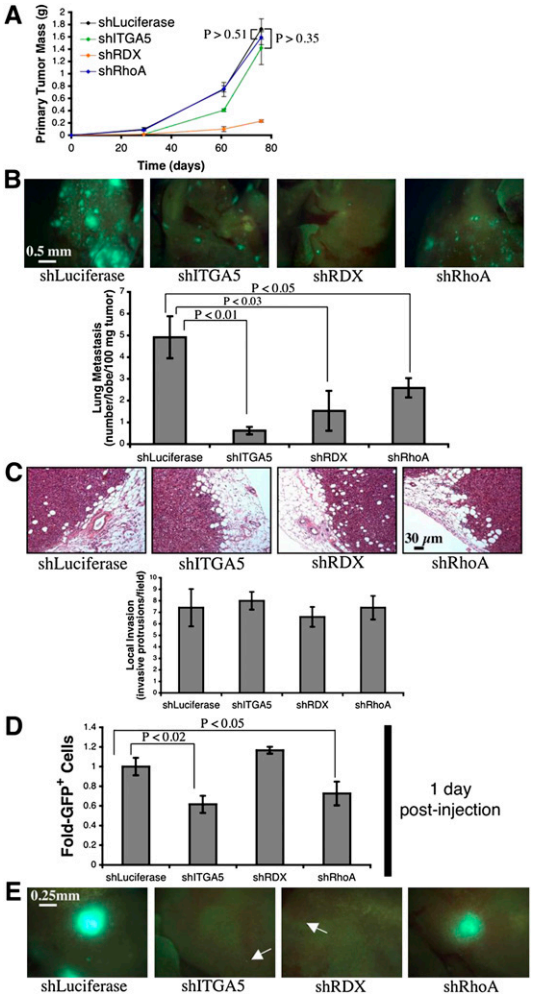

Figure 1. Individual suppression of ITGA5, RDX, or RhoA impairs metastasis in vivo. (*A*) Primary tumor growth upon orthotopic injection of the indicated GFP-labeled 231 cells into NOD/SCID mice. The assay was terminated after 11 wk due to primary tumor burden. $n = 5$ per time point. (*B*, *top* panels) Fluorescent images of murine lungs to visualize 231 cells 76 d after orthotopic implantation. (*Bottom* panel) Quantification of metastatic burden. $n = 5$. (*C*, *top* panels) H&E stain of 231 cell primary mammary tumors 57 d after injection. (*Bottom* panel) Quantification of local invasion. $n = 5$. All *P*-values are >0.67 relative to shLuciferase. (*D*) Prevalence of GFP-labeled 231 cells in the lungs 1 d after intravenous introduction into NOD/SCID mice. $n = 4$. (*E*) Fluorescent images of murine lungs to visualize 231 cells 89 d after intravenous injection. (Arrows) Micrometastases. shRNAs used in these assays were shITGA5 #4, shRDX #3, and shRhoA #5. All error bars represent mean ± SEM.

In our previous work, we observed that miR-31 impinges on three steps of the invasion–metastasis cascade in vivo: local invasion, early post-intravasation events (intraluminal viability, extravasation, and/or initial survival in distant tissues), and colonization (Valastyan et al. 2009). Consequently, we evaluated whether the individual suppression of ITGA5, RDX, or RhoA was sufficient to recapitulate one or more of miR-31's multiple effects on the metastatic process. We found that 231 cells containing shRNAs against either ITGA5, RDX, or RhoA formed primary tumors that appeared histologically invasive and were indistinguishable from controls (Fig. 1C). Thus, inhibition of ITGA5, RDX, or RhoA alone does not abolish local invasion in vivo.

Putative effects on early post-intravasation events were examined by quantifying shRNA-expressing 231 cells in the lungs 1 d after intravenous injection. Cells with either suppressed ITGA5 or RhoA were 40% and 30% less prevalent than controls, respectively; however, RDX knockdown did not reduce persistence in the lungs (Fig. 1D). These effects were not attributable to a differential ability of the cells to become lodged initially in the lung microvasculature, as equal numbers of cells were detected in the lungs 10 min after intravenous injection (Supplemental Fig. 7). These data indicated that inhibition of either ITGA5 or RhoA impairs early post-intravasation events in vivo.

To investigate potential effects on colonization (i.e., the capacity of disseminated single cells to yield large, multicellular metastases), the sizes of lung metastases in intravenously injected animals were analyzed 3 mo after implantation. 231 cells expressing either ITGA5 or RDX shRNAs formed only small micrometastases, while RhoA shRNA-containing cells generated macroscopic metastases comparable with those spawned by control cells (Fig. 1E). Hence, suppression of either ITGA5 or RDX alone prevents colonization in vivo.

Together, these observations revealed that, while individual suppression of ITGA5, RDX, or RhoA impairs one or more steps of the invasion–metastasis cascade, inhibition of any one of these proteins alone is unable to phenocopy the full spectrum of miR-31's impact on metastasis. This suggested that miR-31 may achieve its influences on multiple distinct stages of the metastatic process via concomitant suppression of several downstream effectors. Provocatively, our loss-of-function analyses indicated that ITGA5, RDX, and RhoA act during at least partially distinct steps of the invasion–metastasis cascade (e.g., RhoA affected early post-intravasation events but not colonization, while RDX had no impact on early post-intravasation events but altered colonization); hence, their concurrent regulation provides a plausible mechanism by which miR-31 might elicit its multiple anti-metastatic effects.

To test this hypothesis, we stably re-expressed miRNA-insensitive mRNAs encoding ITGA5, RDX, and RhoA together in combination—along with either miR-31 or control vector—in 231 cells. When these cells were orthotopically injected into mice, miR-31 enhanced primary tumor growth, recapitulating our prior findings (Valastyan et al. 2009); simultaneous re-expression of ITGA5, RDX, and RhoA failed to alter the size of miR-31-containing or control primary tumors (Fig. 2A). Despite their ability to generate larger primary tumors, miR-31-expressing 231 cells were impaired by >80% in their

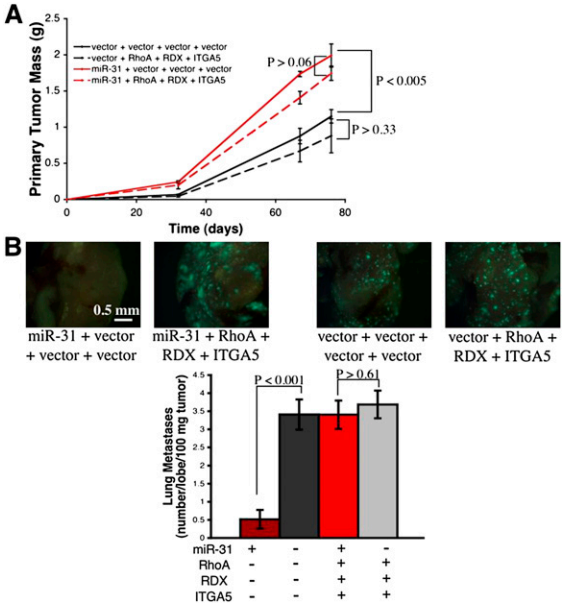

Figure 2. Simultaneous re-expression of ITGA5, RDX, and RhoA abrogates miR-31-imposed metastasis suppression in vivo. (*A*) Primary tumor growth upon orthotopic injection of the indicated GFP-labeled 231 cells into NOD/SCID mice. The assay was terminated after 11 wk due to primary tumor burden. $n = 5$ per time point. (*B*, *top* panels) Fluorescent images of murine lungs to visualize 231 cells 67 d after orthotopic implantation. (*Bottom* panel) Quantification of metastatic burden. $n = 5$. All error bars represent mean ± SEM.

ability to spawn lung metastases (Fig. 2B). ITGA5, RDX, and RhoA did not enhance metastasis in control 231 cells; however, concomitant re-expression of ITGA5, RDX, and RhoA in 231 cells containing miR-31 completely abrogated miR-31-imposed metastasis suppression (Fig. 2B). These data implied that the impact of miR-31 on in vivo metastasis can be explained by miR-31's capacity to inhibit a cohort of three downstream effectors. This was quite surprising, as computational algorithms predict that miR-31 regulates >200 mRNAs, many of which encode proteins that function in metastasis-relevant processes (Krek et al. 2005; Grimson et al. 2007).

Since the combined re-expression of ITGA5, RDX, and RhoA entirely abolished miR-31-evoked metastasis suppression, we also determined whether these three effectors were able to reverse a subset of miR-31's influences on metastasis when re-expressed either individually or in different combinations. Thus, we created 231 cells stably expressing miR-31 or control vector plus all possible permutations of zero, one, two, or three of these miR-31 targets (all rendered miRNA-resistant) (Supplemental Fig. 8). miR-31, ITGA5, RDX, and RhoA failed to affect cell proliferation in vitro (Supplemental Fig. 9A). However, individual re-expression of ITGA5, RDX, or RhoA rescued, at least partially, in vitro defects in invasion, motility, and anoikis resistance conferred by ectopic miR-31; the extent of reversal was more pronounced when multiple effectors were re-expressed in combination (Supplemental Fig. 9B–D).Thus, ITGA5, RDX, and RhoA control in vitro behaviors important for metastasis downstream from miR-31.

To assay the respective abilities of all possible combinations of re-expressed ITGA5, RDX, and/or RhoA to

reverse miR-31's influences on in vivo metastasis, 231 cells expressing miR-31, ITGA5, RDX, and/or RhoA were orthotopically implanted into mice. miR-31 generally promoted primary tumor growth, while restored levels of ITGA5, RDX, and RhoA failed to consistently affect the growth of primary tumors (Fig. 3A; Supplemental Table 1). miR-31 reduced the incidence of metastatic lesions in the lungs by >90% (Fig. 3B). When individually re-expressed in miR-31-containing cells, ITGA5, RDX, or RhoA increased metastasis to 40%, 45%, and 65% of control levels, respectively; re-expression of any two of these targets in miR-31-positive cells yielded 85% as many metastases as controls (Fig. 3B). As before, concomitant re-expression of ITGA5, RDX, and RhoA in cells containing miR-31 restored the number of lung metastases to 100% of that observed in controls (Fig. 3B).

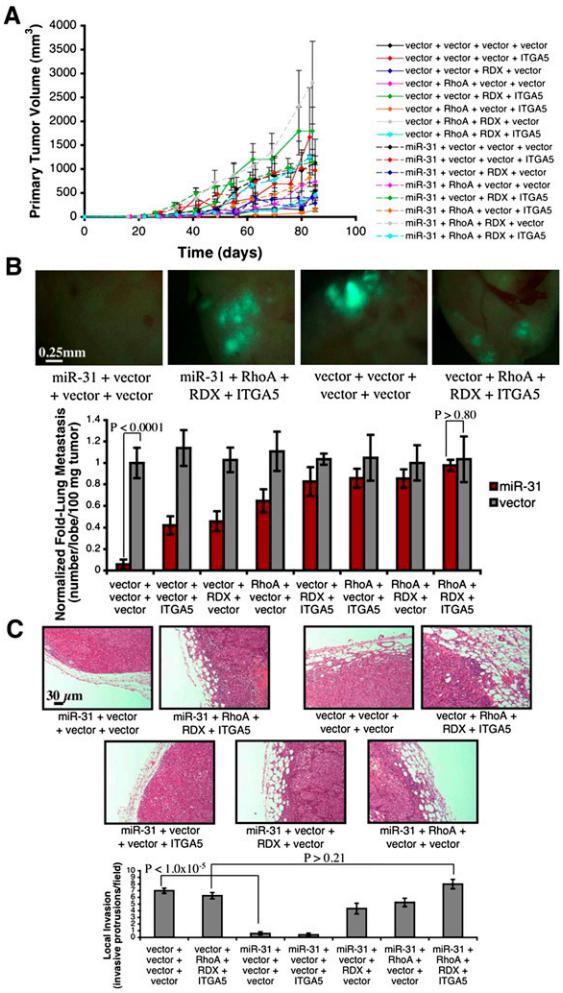

Figure 3. Re-expression of ITGA5, RDX, and/or RhoA affords both unique and partially overlapping reversal of miR-31-evoked inhibition of spontaneous metastasis in vivo. (*A*) Primary tumor growth upon orthotopic implantation of the indicated GFP-labeled 231 cells into nude mice. The assay was terminated after 13 wk due to primary tumor burden. $n = 5$. (*B*,*top* panels) Fluorescent images of murine lungs to visualize 231 cells 88 d after orthotopic injection. (*Bottom* panel) Quantification of metastatic burden. $n = 5$. (*C*) H&E stain of 231 cell primary mammary tumors 54 d after injection. (*Bottom* panel) Quantification of local invasion. $n = 5$. All error bars represent mean ± SEM.

Hence, these three effectors make distinct contributions to in vivo metastasis that can collaborate to explain miR-31's influence on this process; however, these observations failed to delineate the specific step(s) of the invasion–metastasis cascade affected by various combinations of re-expressed ITGA5, RDX, and/or RhoA.

miR-31 affects three steps of the invasion–metastasis cascade in vivo: local invasion, early post-intravasation events, and colonization (Valastyan et al. 2009). To investigate whether ITGA5, RDX, and RhoA—when overexpressed—could synergize to reverse miR-31's effects on local invasion, we examined the histological appearance of primary tumors that developed in orthotopically injected mice. Whereas control 231 cell tumors displayed clear evidence of invasion, miR-31-expressing tumors were well-confined (Fig. 3C), as we documented previously (Valastyan et al. 2009). While ITGA5, RDX, and RhoA did not alter invasion in control 231 cell tumors, combined re-expression of these three targets abolished the previously well-encapsulated phenotype of miR-31-expressing tumors (Fig. 3C). miR-31-containing cells with restored levels of either RDX or RhoA alone formed primary tumors that appeared invasive, although reversal of miR-31-imposed invasion defects was incomplete; ITGA5 did not affect encapsulation (Fig. 3C). These observations revealed that miR-31-dependent attenuation of local invasion can be attributed to miR-31's ability to regulate RDX and RhoA. Ostensibly, in light of our shRNA studies (Fig. 1C), RDX and RhoA function redundantly—with either one another or additional, still-unidentified miR-31 targets—to promote invasion in vivo.

We also examined whether re-expression of these three targets could reverse the impact of miR-31 on early post-intravasation events. To do so, we introduced 231 cells into the venous circulation of mice and assayed the number of cells in the lungs 1 d after injection. Consistent with our previous findings (Valastyan et al. 2009), miR-31-expressing cells were fivefold impaired in their ability to persist in the lungs (Fig. 4A), indicating that miR-31 impeded one or more early post-intravasation events. ITGA5, RDX, and RhoA failed to affect early post-intravasation events in control 231 cells (Fig. 4A). In contrast, individual re-expression of either ITGA5 or RhoA restored the number of miR-31-expressing cells in the lungs to 50% of control levels; RDX did not augment the ability of cells containing miR-31 to persist in the lungs at this time point (Fig. 4A). Simultaneous reintroduction of ITGA5 and RhoA in miR-31-expressing cells sufficed to completely override miR-31-imposed obstruction of early post-intravasation events (Fig. 4A). These effects were not a consequence of an altered ability of ITGA5-, RDX-, RhoA-, and/or miR-31-expressing cells to become lodged initially in the lung microvasculature, as equal numbers of cells were detected in the lungs 10 min after intravenous injection (Supplemental Fig. 10). These data provided evidence that miR-31-evoked suppression of early post-intravasation events can be ascribed to miR-31's ability to modulate ITGA5 and RhoA.

Three months after intravenous injection, control 231 cells generated large macroscopic metastases while miR-31-expressing cells yielded only small micrometastases (Fig. 4B). Hence, miR-31 prevented disseminated tumor cells from reinitiating their proliferative program at the site of metastasis, in consonance with miR-31's reported influence on colonization (Valastyan et al. 2009).

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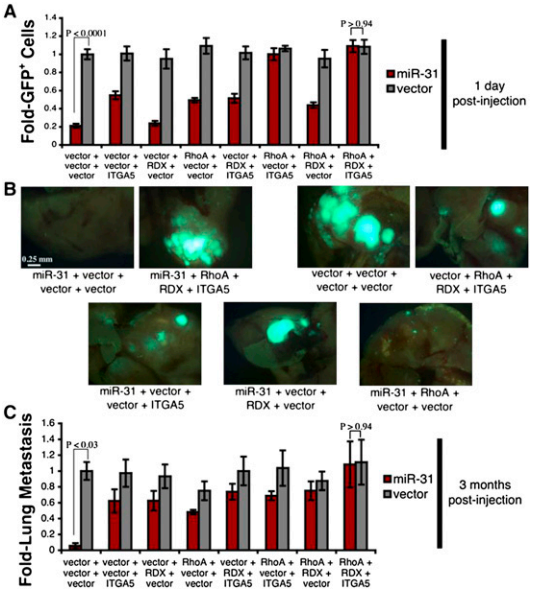

Figure 4. Re-expression of ITGA5, RDX, and/or RhoA affords both unique and partially overlapping reversal of miR-31-mediated inhibition of experimental metastasis in vivo. (*A*) Prevalence of the indicated GFP-labeled 231 cells in the lungs 1 d after intravenous introduction into NOD/SCID mice. $n = 4$. (*B*) Fluorescent images of murine lungs to visualize 231 cells 84 d after tail vein injection. (*C*) Lung metastatic burden 84 d subsequent to intravenous injection. $n = 5$. All error bars represent mean ± SEM.

Concomitant re-expression of ITGA5, RDX, and RhoA in miR-31-containing cells abrogated miR-31-imposed suppression of colonization, yet overexpression of these three targets in control 231 cells failed to increase lesion size (Fig. 4B). Individually restored levels of either ITGA5 or RDX in miR-31-expressing cells reversed miR-31's effects on colonization; RhoA did not affect this parameter (Fig. 4B). Thus, the ability of miR-31 to inhibit colonization can derive from its capacity to suppress ITGA5 and RDX.

In this same assay, miR-31-expressing 231 cells formed 20-fold fewer lung metastases than controls (Fig. 4C). When individually re-expressed in miR-31-containing cells, ITGA5, RDX, or RhoA increased the number of metastases formed to 60%, 60%, and 50% of control levels, respectively (Fig. 4C). Restored levels of pairwise combinations of these three targets in miR-31-expressing cells enhanced lesion number to >70% of controls; importantly, simultaneous re-expression of ITGA5, RDX, and RhoA in miR-31-containing cells completely abolished miR-31-mediated metastasis suppression (Fig. 4C). Taken together, the preceding experiments indicated that the impact of miR-31 on metastasis can be entirely explained by miR-31's capacity to regulate ITGA5, RDX, and RhoA; these three targets act at partially overlapping steps of the invasion–metastasis cascade downstream from miR-31 in vivo (Table 1).

It remained possible that the ability of ITGA5, RDX, and RhoA to override miR-31's actions arose due to some peculiarity of the 231 cell system. To address this, we extended our analyses to SUM-159 human breast cancer cells. Like 231 cells, SUM-159 cells lack endogenous miR-31, are highly aggressive in vitro, and display impaired invasion, motility, and anoikis resistance upon ectopic miR-31 (Valastyan et al. 2009). We created SUM-159 cells stably expressing all 16 potential combinations of either miR-31 or control vector plus miRNA-resistant mRNAs encoding ITGA5, RDX, and/or RhoA; all lines displayed comparable in vitro proliferative kinetics (Supplemental Fig. 11A,B). Consistent with our observations in 231 cells, individual re-expression of ITGA5, RDX, or RhoA in miR-31-containing SUM-159 cells rescued, at least partially, in vitro defects in invasion, motility, and anoikis resistance attributable to ectopic miR-31; as before, the extent of rescue was more pronounced when multiple effectors were concomitantly re-expressed (Supplemental Fig, 11C–E). Hence, the ability of ITGA5, RDX, and RhoA re-expression to override the actions of miR-31 is not confined to 231 cells.

Whereas individual re-expression of ITGA5, RDX, or RhoA largely reversed certain miR-31-imposed metastasis-relevant defects in vitro (Supplemental Figs. 9, 11), individual restoration of ITGA5, RDX, or RhoA levels only partially rescued miR-31's effects on metastasis in vivo (Figs. 3, 4). This underscores the fact that available in vitro assays inadequately model the full complexity of in vivo metastasis; caution must therefore be exercised when deploying these techniques, particularly in the absence of parallel in vivo analyses.

Collectively, the findings of the present study suggest that a miRNA's effects on a given phenotype can be explained by its ability to suppress a relatively modest number of downstream targets. In the present case, the relevant effectors comprise only a small percentage of the total roster of mRNAs targeted by the miRNA under investigation. Our observations are confined to a single miRNA and a single biological endpoint; accordingly, the extent to which this phenomenon is generalizable awaits future investigation. Nevertheless, several recent studies describe strong, but partial, effects on miRNA-mediated phenotypes by modulating individual targets of miRNAs of interest (Ma et al. 2007; Xiao et al. 2007; Yu et al. 2007; Kumar et al. 2008). Such reports suggest the existence of other similarly organized miRNA response networks, in which a miRNA's impact on a biological process can be attributed to that miRNA's ability to inhibit only a small subfraction of its targets.

While our data indicate that ITGA5, RDX, and RhoA represent a minimal cohort of effectors whose regulation is sufficient to account for miR-31's impact on metastasis, these observations do not preclude the existence of additional miR-31 targets that impinge on metastasis-relevant pathways in a manner that ostensibly is functionally redundant with the actions of ITGA5, RDX, and/or RhoA. Also, it is possible that one or more bona fide targets of miR-31 that have metastatic relevance fail to be significantly down-regulated by this miRNA in 231 cells. Overall, due to the fact that metastases are responsible for the overwhelming majority of patient mortality from

Table 1. *Summary of ability of re-expressed targets to rescue miR-31-imposed metastasis suppression*

Target	Local invasion	Early post-intravasation events	Metastatic colonization
ITGA5	No rescue	√	√
RDX	√	No rescue	√
RhoA	√	√	No rescue

Fig(s). 4 live 4/C

carcinomas, this study highlights the idea that modulation of miR-31 and its effectors may prove clinically useful.

Materials and methods

Cell culture

Green fluorescent protein (GFP)-labeled 231 cells have been described (Valastyan et al. 2009). SUM-159 cells were provided by S. Ethier (Ma et al. 2007). Stable expression was achieved via retroviral (expression constructs) or lentiviral (shRNAs) transduction, followed by selection with puromycin, neomycin, hygromycin, and/or zeocin (Elenbaas et al. 2001).

Animal studies

All research involving animals complied with protocols approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care. Age-matched NOD/SCID (propagated on site) or nude (Taconic) mice were used in the xenograft studies, as indicated. For spontaneous metastasis assays, the indicated female mice were bilaterally injected into the mammary fat pads with 1.0×10^6 tumor cells resuspended in 1:2 Matrigel (BD Biosciences) plus normal growth media. In spontaneous metastasis assays employing nude mice, primary tumor diameter was measured every 7 d using precision calipers; tumor volume was calculated according to the formula $V = (4/3)\prod r^3$. For experimental metastasis assays, the indicated mice were injected intravenously with 5.0×10^5 tumor cells (in PBS) via the tail vein. Lung metastasis was quantified using a fluorescent dissecting microscope within 3 h of specimen isolation. Tumor histology was assessed by staining paraffin-embedded tissue sections with hematoxylin and eosin (H&E).

Statistical analysis

Data are presented as mean ± SEM; Student's two-tailed *t*-test was used for comparisons, with $P < 0.05$ considered significant.

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